# The involvement of the neuropeptides orexins (hypocretins) in fear and anxiety in rats exposed to a single episode of footshocks

By

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A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

In partial fulfilment of the requirements of the degree of

**DOCTOR OF PHILOSOPHY**

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# Abstract

Post-traumatic stress disorder (PTSD) is a psychiatric condition that can develop when people experience a stressful and life-threatening event. Clinical research indicates that the presence of a state of hyperarousal after a traumatic experience is the best predictor of a subsequent diagnosis of PTSD. The role of arousal peptides called orexins (hypocretins) in a PTSD-like condition produced by exposing rats to a single episode of footshocks ($5 \times$ 2 s episodes of 1.5 mA) was investigated in this thesis. The first part of my thesis involves the characterization of the footshock model of PTSD and the second part examines the involvement of orexins in this footshock model. The following findings are reported. First, shock rats that exhibited a high level of anxiety to a novel tone (high responders, HR) the day after the footshock exposure subsequently displayed more avoidance when compared to shock rats that exhibited a low level of anxiety (low responders, LR). These results highlight the importance of individual differences in the reaction to a strong fear-inducing experience. Second, the orexin precursor peptide prepro-orexin (ppOX) mRNA was found to be elevated in rats at 6 and 14 days after exposure to footshocks. In addition, ppOX mRNA levels were found to be positively correlated with anxiety at 14 days post-shock. Third, pre-shock injections of the corticotropin releasing factor receptor antagonist antalarmin were found to attenuate the anxiety expressed to the shock chamber and eliminate the correlation between ppOX mRNA levels and anxiety. Fourth, systemic injections of the nonselective orexin receptor antagonist TCS-1102 was found to attenuate the anxiety expressed in rats at 14 days post-shock. Fifth, TCS-1102 was found to have anxiolytic effects that were specific

for the HR.  The results of these experiments provide evidence linking the orexin system

to the anxiety produced by exposure of rats to footshocks.  They also provide preclinical

evidence in support of the use of orexin antagonists for the treatment of anxiety in PTSD.

# Acknowledgements

# Dedication

To my mom, Guorong Chen.

谨将此论文献给我的母亲陈国荣女士。

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# Abbreviations

ABC             avidin-biotin complex

ACTH            adrenocorticotropin

ASD             acute stress disorder

AVP             arginine vasopressin

BNST            bed nucleus stria terminalis

CNS             central nervous system

CRF             corticotropin-releasing factor

CS              conditioning stimulus

DAB             diaminobenzidine

DR              dorsal raphe nuclei

ETM             elevated T-maze

HR              high responder

HPA             hypothalamic-pituitary- adrenal

LC              locus ceruleus

LR              low responder

LSD             least significant differences

mRNA            message ribonucleic acid

OXA               orexin A

OXB               orexin B

$OX_1$ receptor    orexin receptor-1

$OX_2$ receptor    orexin receptor-2

PAG               periaqueductal gray matter

PBS               phosphate buffered saline

PB                phosphate buffer

PEG200            poly-ethylene glycol 200

ppOX              prepro-orexin

PTSD              posttraumatic stress disorder

PVT               paraventricular nucleus of thalamus

RT-PRC            reverse transcription polymerase chain reaction

SSC               sodium chloride-sodium phosphate-EDTA buffer

TMN               tuberomammillary nucleus

US                unconditioned stress stimulus

# Chapter 1

# Introduction

## 1.1. General Introduction

The ability to detect and avoid potential dangers or threats is essential for survival for all organisms. A motor reflex is a basic involuntary response to a stimulus outside the body which may represent a defensive behavior in simple organisms (Purves, Augustine et al. 2011). For example, snails have withdrawal reflexes which allow them to hide in their shells in the presence of danger. Other than basic reflex responses, higher animals have developed emotional responses to deal with the challenges and opportunities in their environment. Consequently, emotions provide a selective evolutionary advantage which is essential for survival of all animals including humans (Marks and Nesse 1994; LeDoux 2012). From the time during which experimental brain research started to flourish in the late 19th century, neuroscientists tried to understand emotions at a functional brain level. However, emotions were considered a mysterious topic for researchers because of their subjective nature. Nonetheless, great progress has been made over the past decades in understanding the neural mechanisms of emotions. For instance, neuroscientists have identified specific brain circuits and neurochemical signals that mediate the basic emotion of fear. In addition, hypotheses have been generated on the potential mechanisms

involved in a number of other emotions including anger, surprise and sadness (Panksepp 2005; Panksepp 2011). The progress that has been made in understanding the neural mechanisms of emotions like fear and anxiety has led to potentially new treatment for some psychiatric conditions in which negative emotions have become disruptive (LeDoux 1992; Davis, Walker et al. 1997; Davis 1998; LeDoux 2000).

Basic emotions like fear and anxiety are easily seen in animals during conditions of danger or threat. Fear is usually produced in the presence of specific predators or objects that pose an immediate danger (Davis, Walker et al. 2010). Another characteristic of fear is that it dissipates quickly when these dangers are no longer present. In contrast, anxiety is evoked by less predictable threats and lingers on after the threat is removed (Davis, Walker et al. 2010). A large body of evidence has accumulated indicating that the neural mechanisms that mediate fear and anxiety are also distinct. For example, the amygdala is the key brain region that is involved in mediating fear whereas the bed nucleus of the stria terminalis is believed to mediate anxiety (Davis 1998; Maren 2001 ). From an evolutionary perspective, the emotions of fear and anxiety are important for survival. Animals with high levels of fear and anxiety may be more likely to detect potential threats and as a consequence escape dangers. For example, it only makes sense that a close encounter between a prey and a predator would produce changes in the brain that allow the prey to be more vigilant in the future so that potential deadly encounter can be avoided. However, if this increase in vigilance generalizes to non-threatening objects and conditions, an animal may be unable to effectively secure food or produce other behaviors necessary for survival. As such, an overactivation of the brain's fear circuitry could have negative consequences for survival of an animal. As will be discussed in this

thesis, post-traumatic stress disorder (PTSD) represents a condition that can develop in people who have experienced an intense fear inducing and stressful event. While the disorder may not directly threaten the survival of a person, PTSD can cause significant distress which will decrease the quality of life of these people.

Individuals that have been exposed to an event involving intense fear, helplessness, or horror usually develop a number of psychological and physiological responses that can vary in terms of intensity and duration. A typical acute reaction to such an event involves a combination of symptoms involving the re-experiencing of the event in the form of memories and flashbacks, nervousness, nightmares, feelings of dissociation, and reluctance to engage in social interactions (Harvey and Bryant 2002; Shalev 2002). For most people, the emotional reactions to a traumatizing event will dissipate over days or weeks, but in some individuals, the reaction does not normalize and can lead to psychological distress and eventually the diagnosis of PTSD (Bonne, Grillon et al. 2004; Davidson, Stein et al. 2004; Stam 2007). As such, PTSD can be considered a disorder of recovery since it results from a failure to recover from a normal negative emotional reaction to traumatic event (Kessler, Sonnega et al. 1995; Yehuda and LeDoux 2007). In these individuals, the negative emotional responses also becomes maladaptive in that the fear and anxiety generalize to situations that bear little resemblance to the original stressful situation (Bonne, Grillon et al. 2004; Stam 2007; Stam 2007; Yehuda and LeDoux 2007). Some researchers have hypothesized that the trauma experience produces changes in the arousability of the brain's fear system which leads to the symptoms that characterizes people who have PTSD (Bonne, Grillon et al. 2004; Stam 2007; Stam 2007). This points to the possibility that the brain's arousal

system change in a way that enhances the expression of fear and anxiety in traumatized individuals.

Orexins (also called hypocretins) are peptides produced exclusively in neurons in the posterior hypothalamus in a bilaterally symmetric organization (de Lecea, Kilduff et al. 1998; Sakurai, Amemiya et al. 1998). These neurons are variable in size (25 - 40 μm in larger diameter) and shape (spherical, mutipolar or fusiform) (Peyron, Tighe et al. 1998; Nambu, Sakurai et al. 1999). It has been estimated that there are about 7000 orexin neurons in the human brain and 3000 in the rat brain (Peyron, Tighe et al. 1998; Sakurai 2007). The region of the lateral hypothalamus in which orexin neurons are found has been associated with appetitive functions such as feeding and drinking (Flier and Maratos-Flier 1998) as well as reward-related behaviors (Harris, Wimmer et al. 2005). This is supported by a series of studies that demonstrated central injection of orexin peptides increases food and water intake (Sakurai, Amemiya et al. 1998; Rodgers, Halford et al. 2000; Thorpe and Kotz 2005). Orexins have also been shown to play an essential role in arousal and maintaining wakefulness (Sakurai 2007; Carter, Adamantidis et al. 2009). The synthesis and the release of orexin peptides in the brain varies in a circadian fashion with high levels found during the active period of organisms' diurnal activity (Taheri, Sunter et al. 2000; Yoshida, Fujiki et al. 2001; Kiyashchenko, Mileykovskiy et al. 2002). Researchers have also found that there is an increase in the activity of orexin neurons during period of wakefulness and exploratory behaviors in rodents (Espana, Plahn et al. 2002; Mileykovskiy, Kiyashchenko et al. 2005; Burgess 2010). On the other hand, a lack of orexin peptides or orexin receptors causes symptoms of narcolepsy such as extreme drowsiness, difficulty of maintaining wakefulness, and

sudden loss of muscle tone while awake (Chemelli, Willie et al. 1999; Nishino, Ripley et al. 2000; Thannickal, Moore et al. 2000; Nishino, Ripley et al. 2001). As such, the orexin system appears to be essential for brain arousal in a variety of situations and conditions.

A number of studies have reported that orexin neurons become more active when animals are exposed to a stressful condition (Ida, Nakahara et al. 2000; Zhu, Onaka et al. 2002; Espana, Valentino et al. 2003; Winsky-Sommerer, Yamanaka et al. 2004; Furlong, Vianna et al. 2009). There is emerging evidence that supports a role for orexins in the regulation of some of the physiological (Kayaba, Nakamura et al. 2003; Furlong, Vianna et al. 2009; Zhang, Kolaj et al. 2010), hormonal (Heydendael, Sharma et al. 2011) and behavioral response (Li, Li et al. 2010) to stressors. A typical stress response includes activation of hypothalamo–pituitary–adrenal (HPA) axis which consists of increased expression and release of corticotropin releasing factor (CRF), adrenocorticotropic hormone (ACTH) and glucocorticoid hormones. Studies have shown that orexin neurons may be activated by stressors through a CRF mechanism (Sakamoto, Yamada et al. 2004; Winsky-Sommerer, Yamanaka et al. 2004; Winsky-Sommerer, Boutrel et al. 2005). Consequently, the interaction between CRF and orexins may represent a pathway by which stress enhances brain arousal levels.

There is increasing evidence that indicates orexins play a role in stress-related highly aroused states such as anxiety, panic and fear (Suzuki, Beuckmann et al. 2005; Li, Li et al. 2009; Johnson, Truitt et al. 2010; Li, Li et al. 2010; Li, Li et al. 2010; Johnson, Samuels et al. 2012; Lungwitz, Molosh et al. 2012; Steiner, Lecourt et al. 2012). For example, central injection of orexin peptides produces anxiety-like behaviors including excessive grooming, avoidance light condition and open spaces (Suzuki, Beuckmann et al.

2005; Li, Li et al. 2009; Li, Li et al. 2010; Li, Li et al. 2010; Heydendael, Sharma et al. 2011; Lungwitz, Molosh et al. 2012), while orexin receptor antagonists have anxiolytic and anti-panic effects (Li, Li et al. 2010; Johnson, Molosh et al. 2012; Johnson, Samuels et al. 2012). Recent studies also link the orexin system to fear by showing that a dual orexin receptor antagonist reduces cardiac responses of fear (Furlong, Vianna et al. 2009) and fear-potentiated startle reactions (Steiner, Lecourt et al. 2012). Fear-potentiated startle is a behavioral response used to quantify fear by measuring the amplitude of acoustic startle reflex in response to a discriminative stimulus associated previously with a shock (Brown, Kalish et al. 1951; Davis 1992). People with PTSD often shows heightened startle to acoustic stimuli (Butler, Braff et al. 1990; Jovanovic, Norrholm et al. 2009) and exaggerated fear potentiated startle responses (Jovanovic, Norrholm et al. 2009; Glover, Phifer et al. 2011). Taken together, evidence from multiple sources indicates that orexins released in the brain mediate stress-like emotions involving fear and anxiety. Evidence also suggests that CRF may play an important role in activating the orexin system. Consequently, the activation of the orexin system by stress may produce highly aroused negative emotional states and represents a potential mechanism involved in the development of PTSD.

## 1.2. Post-traumatic Stress Disorder (PTSD)

### 1.2.1. Symptoms and Progression

Post-traumatic stress disorder (PTSD) is a disabling psychiatric condition that can be triggered in individuals that experience a traumatic event involving serious injury or near-death (for example, individuals exposed to combat warfare, terrorist acts, and sexual assault). The disorder is characterized by three clusters of symptoms that include the re-experiencing (distressing images, flashbacks, nightmares and physiological responses to cues of the events); avoidance/numbing (avoid persons, places or thoughts associated with the event, restricted emotions and less interest in normal activities); and hyperarousal (insomnia, irritability, hypervigilance, and increased startle response) (Yehuda 2002; Shalev 2009; Bisson 2010). The diagnosis of PTSD requires that the symptoms be present for more than one month for acute PTSD and for three months for chronic PTSD (1994). Longitudinal studies also show that PTSD may have a fluctuating course with the symptoms of the disorder often triggered by reminders of the traumatic event or exposure to other life stressors (Yehuda 2002; Shalev 2009; Bisson 2010). The condition will be diagnosed as delayed onset PTSD if the symptoms develop at least 6 months after the stressors (1994). The duration of the symptoms in PTSD patients varies in different populations. In some cases, the symptoms of PTSD can last for decades, even a lifetime (Kessler, Sonnega et al. 1995; Van Ameringen, Mancini et al. 2008).

In the nineteenth century, the idea that people frequently show distressing psychological reactions after exposures to traumatic events became generally accepted.

The Diagnostic and Statistical Manual of Mental Disorders (DSM III) introduced PTSD as a distinct psychiatric disorder in the year of 1980 (1980; Turnbull 1998). Since then, nationwide surveys in North America and Europe have been conducted to study the prevalence of this disorder and have led to the understanding that exposure to trauma appears to be a relatively common occurrence. Kessler et al. reported that the probability of an exposure to at least one traumatic event in the USA is about 60% in the general population during a lifetime (Kessler, Sonnega et al. 1995; Breslau, Kessler et al. 1998), and the lifetime prevalence of PTSD was shown to range from 8% to 10% (Kessler, Sonnega et al. 1995; Hidalgo and Davidson 2000). The current data on PTSD population in Canada is limited. Some regional studies have suggested that most individuals in Canada (about 70%) have been exposed to at least one traumatic event in their lifetime (Stein, Walker et al. 1997; Perkonigg, Kessler et al. 2000). According to an epidemiological study, an estimated prevalence rate of lifetime PTSD in Canada was found to be 9.2% (Van Ameringen, Mancini et al. 2008).

The symptoms of PTSD often overlap with other psychiatric conditions. For example, hypervigilance and increased startle reflex are also included in the symptoms of generalized anxiety disorder (Hoge, Ivkovic et al. 2012). Emotional numbness and sleep problems are also core symptoms of major depressive disorder (Soleimani, Lapidus et al. 2011). In addition, there is growing evidence that PTSD increases the risk of other physical and mental illnesses (Schnurr and Jankowski 1999). Clinical studies have shown that more than half of PTSD patients are reported to have at least one comorbid diagnosis of other psychiatric disorders (Kessler, Sonnega et al. 1995) including depression, mania, generalized anxiety disorder, panic disorder, social phobia, alcohol or

drug abuse, and suicidal tendencies (Kessler, Sonnega et al. 1995; Brady, Killeen et al. 2000; Davidson, Stein et al. 2004; Kessler, Avenevoli et al. 2012). The high rates of comorbidity with other psychiatric illness along with persistent symptoms increases the difficulty associated with treating PTSD. Consequently, PTSD is associated with huge health care use and economic cost (Solomon and Davidson 1997; Kessler 2000; Marshall, Jorm et al. 2000; Deykin, Keane et al. 2001).

## 1.2.2. Individual Differences in PTSD

Studies that follow people after exposure to a severe trauma indicate that most individuals will express some PTSD-like symptoms shortly after the traumatic event (Peleg and Shalev 2006). While in most cases these symptoms will dissipate over weeks, in some individuals the symptoms will persist and sometimes intensify over time (Peleg and Shalev 2006). The relatively low prevalence of PTSD among individuals that experienced traumatic events raises the question of what factors aside from trauma are required for PTSD to develop. Most researchers that have tried to identify some of the individual difference that led to PTSD have focused on the contributing factors of gender, age, race, education level, personality traits, psychiatric history and trauma type (Breslau, Davis et al. 1995; Hidalgo and Davidson 2000; Stam 2007). Interestingly, several studies have shown that patients with early diagnosis of Acute Stress Disorder (ASD) have a much higher probability for a diagnosis of PTSD later (Bryant and Harvey 1998; Harvey and Bryant 1998; Bryant, Harvey et al. 2000). ASD has been defined as the acute response (re-experiencing, avoidance, and hyperarousal) that occurs during two days to

four weeks after trauma event (1994; Cardena and Carlson 2011). This classification attempts to bridge the gaps between the acute and chronic response to trauma. The fact that people with ASD have a high chance of developing PTSD indicates that similar neural mechanisms may be responsible for ASD and PTSD.

Clinical researchers have studied the temporal progression of trauma-related distress symptoms and they have found a different developmental course for the symptoms. The re-experiencing symptoms generally appear early after trauma but then start to decline over time (Schell, Marshall et al. 2004) whereas the avoidance symptoms normally happen later and often determine the diagnosis of PTSD (North 2001). On the other hand, hyperarousal symptoms tend to precede and predict the subsequent development of the other symptoms (Weems, Saltzman et al. 2003; Schell, Marshall et al. 2004; Thompson, Vasterling et al. 2004; Feuer, Nishith et al. 2005; Marshall, Schell et al. 2006; Solomon, Horesh et al. 2009). A persistent hyperarousal state is often observed in people with ASD or PTSD. Consequently, it is possible that increased baseline level of brain arousal plays an essential role in ASD and contributes to the development of PTSD later.

It is well recognized that generalized states of arousal are important for normal brain function and that different forms of arousal states involving hunger, thirst, sex, fear and pain promote specific behaviors that are related to these states (Garey, Goodwillie et al. 2003; Pfaff, Westberg et al. 2005; Pfaff and Banavar 2007; Pfaff, Ribeiro et al. 2008). Since generalized arousal plays an important role in fundamental cognitive and emotional functions, malfunction of arousal may lead to a wide range of health problems. A stressful experience may sensitize the arousal centers in the brain and lead to persistent

state of hyperarousal. Hyperarousal may influence other regions of the brain that regulate negative emotions and avoidance (Frewen and Lanius 2006), and most likely contributes to the other symptoms of ASD and PTSD (Feuer, Nishith et al. 2005; Marshall, Schell et al. 2006). Additionally, a hyperaroused state may enhance the expression of other symptoms of PTSD. It is known that PTSD has a high comorbidity with other psychiatric disorders. The changes in the arousal centers of the brain may also lead to the subsequent development of depression and other anxiety disorders. As such, a plausible link exits between the hyperarousal effects produced by exposure to a severely traumatizing situation and the distressing, numbing, and avoidance associated with PTSD.

### 1.2.3. Fear and PTSD

The word "fear" most commonly refers to peoples' conscious feelings in people of the presence of a threat to their well-being. From an experimental neurobehavioral perspective, the word "fear" is also used to refer to the behavioral and physiological responses that are elicited by threats (LeDoux 2013). The responses can be quantified in animals to establish the presence of the emotion we normally associate as being related to our conscious feeling of fear. Fear behaviors in humans and other mammals include phasic immobility (freezing), alteration in autonomic nervous system, increased reflexive responses to sensory stimuli (e.g. startle), hypoalgesia, and increased urination and defecation (Blanchard and Blanchard 1988). It has been proposed that an exaggerated fear state is the root of the pathological anxiety (Rosen and Schulkin 1998). Thus, the

study of the neuronal mechanism of fear is important for understanding the anxiety disorders such as PTSD.

During a traumatic event, most people feel intense fear, helplessness or horror. These strong emotional reactions are strongly associated with external cues or places and stored as memories in the brain. Thus, the cues of the trauma remind people of the original stressful experience and cause excessive fear response when memories of the situation are remembered. The re-experiencing symptom cluster of PTSD is viewed as resulting from fear conditioning (Shalev, Ragel-Fuchs et al. 1992). In psychological studies, Pavlovian fear conditioning is defined as a process of learning where a neutral conditioning stimulus (CS) comes to elicit a conditioned fear response (CR) after being associated with an unconditioned stress stimulus (US). For example, after exposure to footshocks (US) paired with an acoustic tone (CS), rats express increased immobility (fear response) to the tone in the absence of footshocks (conditioned fear) as well as to the chamber where rats received footshocks (contextual fear) (Baldi, Lorenzini et al. 2004). Pavlovian fear conditioning is viewed as an adaptive mechanism with evolutionary benefits for survival (Rosen and Schulkin 1998). When stressors become more intense, fear generalization and sensitization become more prominent. Fear generalization is a phenomenon in which stimuli or conditions resembling the original CS come to elicit a fear response similar to that elicited by the CS (Radulovic, Kammermeier et al. 1998). On the other hand, exposure to extremely fear-inducing situations can produce an enhanced fear response to neutral stimuli and conditions (stimuli that do not resemble the CS or US) in a process called fear sensitization (Kamprath and Wotjak 2004; Daviu, Fuentes et al. 2010). For instance, rodents that previously exposed to footshocks

also show increased immobility to the novel chamber (fear generalization) and novel subjects (fear sensitization) (van Dijken, Mos et al. 1992; Van Dijken, Tilders et al. 1992; Louvart, Maccari et al. 2005; Louvart, Maccari et al. 2006; Siegmund and Wotjak 2007; Mikics, Baranyi et al. 2008).

Fear conditioning (Mineka and Zinbarg 2006), generalization (Lissek, Biggs et al. 2008; Greenberg, Carlson et al. 2012) and sensitization (Corley, Caruso et al. 2012) have been implicated in the development and maintenance of pathologic anxiety disorders. Clinical evidence clearly shows that individuals exposed to severe trauma subsequently exhibited fear and anxiety when confronted with reminders of the situation as well as novel situations not directly related to the trauma (Charney, Deutch et al. 1993; Stam, Bruijnzeel et al. 2000). Similarly, rats that were previously exposed to footshocks not only showed a strong fear response when re-exposed to the shock apparatus or to cues associated with the shocks (fear conditioning), but also displayed hypervigiliance, fear and anxiety when exposed to novel environments or sounds (fear generalization and sensitization) (van Dijken, Mos et al. 1992; Van Dijken, Tilders et al. 1992; Louvart, Maccari et al. 2005; Louvart, Maccari et al. 2006; Siegmund and Wotjak 2007; Mikics, Baranyi et al. 2008). Rodents pre-exposed to footshocks also exhibited a decrease in social interaction with conspecifics and avoidance of areas of mazes involving some level of novelty or unknown risk (van Dijken, Mos et al. 1992; Bruijnzeel, Stam et al. 2001; Bruijnzeel, Stam et al. 2001; Louvart, Maccari et al. 2005; Mikics, Baranyi et al. 2008; Mikics, Toth et al. 2008). These observations provide evidence that fear can generalize to a variety of situations, and that hyperactivity of the fear systems may also contribute to the symptoms of PTSD.

With multiple exposures to the CS, animals begin to learn that the CS no longer predicts the US and start to show decreased conditioned or generalized fear response. This learning process is referred as fear extinction (Herry, Ferraguti et al. 2010). Fear extinction is not simply forgetting the traumatic experience but a process of re-relearning (Myers and Davis 2007) which involves the hippocampus (Vianna, Coitinho et al. 2004; Milad, Wright et al. 2007). Impaired extinction of fear memories is thought to contribute to the development and persistence of anxiety disorders such as PTSD (Anderson and Insel 2006; Rauch, Shin et al. 2006; Milad, Orr et al. 2008). Therefore, PTSD can be viewed as a anxiety disorder which involves changes in the fear circuits and deficits in extinction learning (Friedman, Resick et al. 2011; Zoellner, Rothbaum et al. 2011).

### 1.2.4. HPA Axis and PTSD

The hypothalamic-pituitary-adrenal (HPA) axis plays an essential role in helping organisms adapt to homeostatic challenges in response to stressors. Activation of the HPA axis starts with increased secretion of a neuropeptide called corticotropin-releasing factor (CRF) in the hypothalamus. Once CRF reaches the anterior pituitary, it triggers the release of adrenocorticotropin (ACTH) into the circulation. The adrenal gland receives the ACTH signal and releases glucocorticoid hormones (mainly cortisol in humans and corticosterone in rodents) to prepare the body for its response to a threatening situation. In addition, the glucocorticoid hormones provide negative feedback inhibition of hypothalamus and pituitary to slow down the synthesis and release of CRF and ACTH (Herman, Figueiredo et al. 2003; Abelson, Khan et al. 2010). Within

the HPA axis, CRF is the principal regulator of endocrine responses to stressors in the central nervous system. Other than its participation in the activation of HPA axis, CRF is also involved in the expression of fear and anxiety. A small population of neurons in the central nucleus of amygdala also produce CRF which is believed to modulate fear and anxiety through actions on fear circuits in the brain (Adamec, Sayin et al. 1991; Kumar and Karanth 1996; Radulovic, Ruhmann et al. 1999; Kikusui, Takeuchi et al. 2000; Radulovic, Fischer et al. 2000; Zorrilla, Valdez et al. 2002; Lehner, Taracha et al. 2008; Bijlsma, van Leeuwen et al. 2011). Studies have demonstrated that central injection of CRF into the brain enhances fear behaviors and the retrieval fear memory (Kumar and Karanth 1996; Radulovic, Fischer et al. 2000; Skorzewska, Bidzinski et al. 2008; Borelli, Albrechet-Souza et al. 2013). In contrast, treatment with CRF receptor antagonists produces the suppression of fear responses and fear memory (Kumar and Karanth 1996; Deak, Nguyen et al. 1999; Hikichi, Akiyoshi et al. 2000). In addition, a number of studies indicate that CRF contributes to anxiety-like behaviors. For example, central injection of CRF produced anxiogenic-like effects in rodents (Koob and Bloom 1985; Dunn and Berridge 1990; Dunn and Berridge 1990), and blocking of CRF receptors attenuated anxiety displayed when rodents were exposed to the elevated plus-maze (Lundkvist, Chai et al. 1996), light-dark box (Griebel, Perrault et al. 1998), and defensive withdrawal box (Arborelius, Skelton et al. 2000). The effects of CRF are mediated via binding to two types of receptors: the CRF receptor-1 and CRF receptor-2. The CRF receptor-1, but not the CRF receptor-2, has been shown to play important roles in regulating fear- and anxiety-related behavior (Lundkvist, Chai et al. 1996; Heinrichs, Lapsansky et al. 1997; Gresack, Risbrough et al. 2010). It is possible that CRF

contributes to the symptoms of PTSD since hyperexcitability of fear and anxiety are key components of PTSD.

Clinical observations have demonstrated altered HPA axis acitivity in PTSD patients (Yehuda 2001; de Kloet, Vermetten et al. 2006). This includes a combination of high baseline levels of CRF in cerebrospinal fluid and low plasma cortisol levels (Yehuda 1997; Yehuda 2001; Yehuda 2005; de Kloet, Vermetten et al. 2006). Several theories have been suggested to explain this phenomenon. First, it is proposed that increased sensitivity of glucocorticoid receptor in the anterior pituitary gland leads to enhanced binding of cortisol, which results a negative feedback inhibition of ACTH and cortisol production and release (Newport, Heim et al. 2004; Yehuda, Golier et al. 2004). In addition, the low plasma levels of cortisol stimulate more release of CRF from the hypothalamus in order to normalize cortisol levels (Yehuda, Levengood et al. 1996). Inhibition of cortisol levels in PTSD patients may also result from insufficient pituitary function in response to low cortisol levels. Similarly, malfunction of the adrenal glands or reduced sensitivity of the adrenals for ACTH could also result in enhanced suppression of cortisol levels (Yehuda, Levengood et al. 1996; Kanter, Wilkinson et al. 2001). Another hypothesized mechanism involves alterations in the interaction with cortisol binding peptides. One study has assessed the plasma levels of glucocorticoid binding globulin in PTSD patients and has reported that the binding globulin is higher in PTSD than control subjects (Kanter, Wilkinson et al. 2001). The increased level of glucocorticoid binding globulin would limit the availability of cortisol and the negative feedback of cortisol on ACTH secretion (Berdusco, Yang et al. 1995). Those potential

mechanisms may lead to increased CRF levels but decreased coritsol levels in people with PTSD.

The increased level of CRF is found in people with PTSD. Therefore, blocking CRF receptors may help relieve the symptoms of PTSD. Indeed, animal studies have shown that rats that received the CRF receptor-1 antagonism before the shock exposure display reduced contextual fear response to the shock chamber (Hubbard, Nakashima et al. 2007). The CRF receptor antagonism also has been shown to prevent the initiation and consolidation of anxiety-like behaviors in a mouse predator stress model of PTSD (Adamec, Fougere et al. 2010). These animal studies support further research of CRF receptor antagonism as a potential prophylactic treatment for clinical PTSD.

### 1.2.5. Exposure Therapy and Pharmacological Treatments for PTSD

As mentioned earlier, fear extinction happens when the conditioned stimulus is no longer associated with an aversive unconditioned stimulus in a way that the contextual fear becomes attenuated. As PTSD is believed to be a psychiatric disorder involving an over-expression of fear, the principle of fear extinction is applied for treating people with PTSD. The clinical procedure of repeatedly exposing patients with fearful objects, or situations with no real danger present is called "exposure therapy" (Rauch, Eftekhari et al. 2012). The purpose of exposure therapy is to have PTSD patient relearn that the conditioned stimuli that previously predicted the aversive condition are no longer potential threats. This extinction procedure is designed to reduce fear, anxiety, and

arousal levels in patients (Foa, Hembree et al. 2007).  Clinical evidence also supports the idea that exposure therapy helps to manage the symptoms of PTSD in some people (Marks, Lovell et al. 1998; Foa, Dancu et al. 1999; Rauch, Eftekhari et al. 2012).

Fear extinction does not erase the memory of trauma experience and the feelings associated with fear and horror.  The conditioned fear response may persist even after extinction training, and fear may reappear when individuals confront novel stressful conditions (Myers and Davis 2002; Davis, Myers et al. 2006).  Similarly, clinical observations also have shown that exposure therapy has little or no effect on PTSD symptoms and can even exacerbate the symptoms in some individuals (Pitman, Altman et al. 1991; Devilly and Foa 2001).  Since PTSD is highly comorbid with other psychiatric disorders (Kessler, Sonnega et al. 1995), exposure therapy may result in additional aversive emotional responses that contribute to other disorder (Hagenaars, van Minnen et al. 2010).  Exposure therapy is controversial and further research is needed to verify its clinical effectiveness.

The pharmacological treatments for PTSD require an understanding of the neural circuits and substrates associated with fear learning, adaptation to severe stressors, sensitization and generalization of fear responses, and fear extinction learning.  The serotonergic system is known for playing a role in the regulation of stress and anxiety (Griebel 1995; Harvey, Naciti et al. 2004).  It has been proposed that dysfunctions in the serotonergic system may be involved with PTSD (Lee, Lee et al. 2005; Krystal and Neumeister 2009; Xie, Kranzler et al. 2009).  Selective serotonin reuptake inhibitors (SSRIs) are effective in reducing symptom severity and preventing relapse in some PTSD patients (van der Kolk, Dreyfuss et al. 1994; Connor, Sutherland et al. 1999; Davidson,

Rothbaum et al. 2006; Onder, Tural et al. 2006).  Thus, SSRIs are used as the first-line

drug treatment for PTSD.  However, the use of SSRIs is questionable because of their

lack of efficacy, delayed onset of action, and undesirable adverse effects in some patients

(e.g. cardiovascular and sexual dysfunction) (Stein, Kline et al. 2002; Zohar, Amital et al.

2002).  Other neuropeptides or neurotransmitters are also likely to be involved in

pathology of PTSD.  For example, the activity of noradrenergic neurons in PTSD patients

may contribute to hyperarousal symptoms (Southwick, Bremner et al. 1999).

Normalizing noradrenergic neuron hyperactivity is also a potential target for treating

PTSD.  Indeed, clinical studies have shown that blocking of noradrenergic receptors

improves hyperarousal symptoms including the sleep disturbance associated with PTSD

(Peskind, Bonner et al. 2003; Taylor, Martin et al. 2008).  However, these drugs are not

effective in reducing other symptoms.  A number of other pharmacological approaches

including the use of antidepressants, anxiolytics or antipsychotics have been used but

have been reported to have limited effects (Davis, Frazier et al. 2006; Zhang and

Davidson 2007; Shalev 2009).  The poor efficacy of the pharmacological agents available

to treat PTSD points to the need for more effective therapeutic approaches.


## 1.2.6. Footshock Model of PTSD


In 1872, Charles Darwin proposed the ground-breaking observations that humans

and other animals share fundamental emotions including fear, anger, surprise and sadness

(Darwin 1872/1965).  These observations by Darwin and other scholars led to the idea

that using animal models to study the mechanisms of basic emotion to understand

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emotional disorders was a valuable approach. Animal models of neuropsychiatric disorders represent an essential tool for understanding the mechanisms involved in these disorders and for developing pharmacological tools to treat them. Examples of animal models for PTSD include exposure of rodents to single or repeated episodes of electrical shock, predators or other stressors (Stam, Bruijnzeel et al. 2000; Wiedenmayer 2004; Siegmund and Wotjak 2006; Cohen, Kozlovsky et al. 2012). These stressors can produce long-lasting conditioned fear responses to trauma-related cues and generalized/sensitized fear response to neutral stimuli.

Similar to the effect of a severe stress on people, exposure of rodents to a brief episode of relatively intense footshocks causes these rats to show fear and anxiety to novel chambers, tones, and objects in addition to the fear associated with the shock chamber (van Dijken, Mos et al. 1992; Van Dijken, Van der Heyden et al. 1992; Bruijnzeel, Stam et al. 2001; Bruijnzeel, Stam et al. 2001; Louvart, Maccari et al. 2005; Louvart, Maccari et al. 2006; Siegmund and Wotjak 2007; Mikics, Baranyi et al. 2008; Mikics, Toth et al. 2008). Exposure of rodents to inescapable electrical shock appears to be one of the most common and reliable means of producing animals that express a PTSD-like condition (van Dijken, Mos et al. 1992; Louvart, Maccari et al. 2005; Siegmund and Wotjak 2007; Mikics, Baranyi et al. 2008). Components of each of the symptom clusters can be operationally defined and demonstrated to last over weeks or months. For example, increases in startle responses and hypervigiliance (hyperarousal cluster) have been reported (van Dijken, Mos et al. 1992; Van Dijken, Tilders et al. 1992; Pynoos, Ritzmann et al. 1996; Mikics, Baranyi et al. 2008). Increases in fear or anxiety towards objects or conditions that were present at the time of the stressor (re-experiencing

symptoms) have also been documented (Louvart, Maccari et al. 2006; Siegmund and Wotjak 2007; Mikics, Baranyi et al. 2008). Additionally, increases in anxiety to a novel environment and noises (van Dijken, Mos et al. 1992; Van Dijken, Tilders et al. 1992; Louvart, Maccari et al. 2005; Siegmund and Wotjak 2007), decreases in social interaction with other rodents, (Louvart, Maccari et al. 2005; Mikics, Baranyi et al. 2008; Mikics, Toth et al. 2008) and increases in avoidance (avoidance/numbing cluster) (van Dijken, Mos et al. 1992; Bruijnzeel, Stam et al. 2001; Bruijnzeel, Stam et al. 2001; Louvart, Maccari et al. 2005) have been demonstrated by a number of laboratories. It is apparent from a number of studies that the development of PTSD-like behaviors in pre-shocked rodents is dependent on the intensity of the shock exposure (Baldi, Lorenzini et al. 2004; Rau, DeCola et al. 2005; Mikics, Baranyi et al. 2008; Rau and Fanselow 2009). Studies also show that individual differences in fear responses of shock rodents can account for some of the variability in the fear learning that can occur after the shock exposure (Rau, DeCola et al. 2005). This is similar with a clinical condition that shows acute trauma responses predict the later development of PTSD (Bryant and Harvey 1998; Harvey and Bryant 1998; Bryant, Harvey et al. 2000). Therefore, footshock in rodents provides a model to study neural mechanisms, and potential pharmacological treatments for PTSD.

## 1.3. Orexins in the Regulation of Arousal, Stress, and Negative Emotions

### 1.3.1. Orexin Peptides, Receptors, and Distribution

PTSD can be viewed as an anxiety disorder displaying exaggerated fear expression and impaired fear extinction. An increase in brain arousal levels may contribute to the generalized and sensitized fear responses and the development of symptoms in PTSD. Thus, the study of the brain mechanisms of arousal which contribute to the hyperarousal produced by a traumatic experience may lead to potential pharmacological targets for treating PTSD. Orexin peptides were discovered in 1998 by two independent research groups (de Lecea, Kilduff et al. 1998; Sakurai, Amemiya et al. 1998). A wide range of experimental evidence shows that orexins are arousal promoting peptides which regulate behavioral arousal states (Ganjavi and Shapiro 2007; Nishino 2007; Carter, Borg et al. 2009; Tsujino and Sakurai 2009; Sakurai, Mieda et al. 2010). As such, orexin system represents an excellent candidate for producing the hyperarousal symptoms associated with PTSD.

Orexins are peptides produced exclusively in neurons located in the posterior hypothalamus. The bioactive peptides orexin-A (OXA) and orexin-B (OXB) are cleaved from the precursor peptide prepro-orexin (ppOX) (de Lecea, Kilduff et al. 1998; Sakurai, Amemiya et al. 1998). OXA is a 33-amino-acide peptide of 3562 Da with two sets of intrachain disulfide bonds, whereas OXB is a linear peptide with 28-amino-acide of 2937 Da, and share 46% identical sequence with OXA (de Lecea, Kilduff et al. 1998; Sakurai,

Amemiya et al. 1998).  Both of OXA and OXB peptides have highly conserved structures

between different species such as human, rodent, chicken, xenopus and fish (Shibahara,

Sakurai et al. 1999; Ohkubo, Boswell et al. 2002; Kaslin, Nystedt et al. 2004), indicating

that the physiological role of orexins is well-maintained during evolution.  Orexins act at

two G-protein coupled receptors called orexin receptor-1 ($OX_1$ receptor) and orexin-2

receptor ($OX_2$ receptor) (de Lecea, Kilduff et al. 1998; Sakurai, Amemiya et al. 1998).

$OX_1$ receptor is found to be coupled exclusively to the Gq subclass of heterotrimeric G

proteins, whereas $OX_2$ receptor can bind to Gi/o and/or Gq subclass (Sakurai, Amemiya

et al. 1998).  Competitive radioligand binding assays have demonstrated that OXA and

OXB have the same affinity for $OX_2$ receptor, whereas OXB has lower affinity for $OX_1$

receptor compared to OXA (Sakurai, Amemiya et al. 1998).

Orexin neurons send their fibers to multiple areas of the brain with the heaviest

orexin-immunoreactive nerve endings being found in the bed nucleus stria terminalis

(BNST), amygdala, paraventricular nucleus of thalamus (PVT), arcuate nucleus of the

hypothalamus, dorsal raphe nuclei (DR), tuberomammillary nucleus (TMN), and locus

ceruleus (LC) (Peyron, Tighe et al. 1998; Cutler, Morris et al. 1999; Date, Ueta et al.

1999; Nambu, Sakurai et al. 1999).  Those brain areas are shown to regulate arousal,

feeding, reward and stress.  Orexin neurons also receive inputs from the BNST, amygdala,

lateral septum, dorsomedial nucleus of hypothalamus, posterior hypothalamus,

periaqueductal gray matter (PAG), DR, and lateral parabrachial nucleus (Sakurai, Nagata

et al. 2005; Yoshida, McCormack et al. 2006), areas which are related with arousal, stress,

anxiety, fear and pain.  The anatomical connections of orexin neurons suggest that

orexins have wide ranging influences on the brain.

## 1.3.2. Orexins in the Regulation of Arousal

Many researchers have shown that orexins play important roles in maintaining behavioral arousal (Ganjavi and Shapiro 2007; Nishino 2007; Carter, Borg et al. 2009; Tsujino and Sakurai 2009; Sakurai, Mieda et al. 2010). Anatomical studies illustrate that orexin neurons send their projections to nuclei that produce arousal including the PVT, LC (containing noradrenergic neurons), DR (which contains serotonergic neurons) and TMN (containing histaminergic neurons) (Peyron, Tighe et al. 1998; Date, Ueta et al. 1999). Microinjections of orexins into the brain were shown to prolong the duration of wakefulness and to produce behavioral activation including food intake, drinking, and locomotor activity (Nakamura, Uramura et al. 2000; Rodgers, Halford et al. 2000; Espana, Plahn et al. 2002). On the other hand, loss of orexin signals in the brain results in the narcoleptic phenotype in mice and dogs (Chemelli, Willie et al. 1999; Lin, Faraco et al. 1999), which is characterized by a difficulty in maintaining wakefulness. Clinical evidence also shows that there is a loss of orexin neurons in the brain of humans with narcolepsy (Nishino, Ripley et al. 2000; Peyron, Faraco et al. 2000; Thannickal, Moore et al. 2000; Ripley, Overeem et al. 2001; Crocker, Espana et al. 2005). Consistent with a role in arousal, orexin neurons have been shown to be more active during period of wakefulness (Estabrooke, McCarthy et al. 2001; Espana, Valentino et al. 2003; Lee, Hassani et al. 2005). Electrophysiological recordings done in free-moving rats demonstrate that orexin neurons are especially active when rats are alert and moving (Mileykovskiy, Kiyashchenko et al. 2005). In addition, a considerable amount of evidence has accumulated in support of a role for orexins in the regulation of appetitive

and reward-seeking behaviors (Rodgers, Ishii et al. 2002; Harris and Aston-Jones 2006; Boutrel and de Lecea 2008; Di Sebastiano and Coolen 2012). As a whole, these observations indicate that orexins regulate arousal levels associated with a number of different behavioral states.

### 1.3.3. Orexins in the Regulation of Stress

Stress has been defined as a behavioral state elicited by challenging or threatening situations that disrupt homeostasis (Selye 1946). Physiological responses to stressor include activation of catecholamine systems (noradrenaline and adrenaline) and the HPA axis. These reactions are believed to help animals adapt to challenging and dangerous situations (Selye 1946). A key aspect of stress is a heightened level of arousal and readiness for action, which may alter a variety of state-dependent processes including attention, memory, and sensory information processing (Arnsten, Berridge et al. 1985; Berridge and Dunn 1989; Arnsten 1999). As such, a sustained level of arousal is an important feature of the stress response. Neuropeptides that are involved in regulating arousal states may contribute to the pathological process involved in PTSD.

Orexins are well known for their roles in the maintenance of arousal. Recent evidence suggests that orexins may also be involved in stress reactions. A number of studies using cFos as marker of a neuronal excitation have reported that orexin neurons become more active when animals are exposed to a stressful condition (Ida, Nakahara et al. 2000; Zhu, Onaka et al. 2002; Espana, Valentino et al. 2003; Winsky-Sommerer,

Yamanaka et al. 2004; Furlong, Vianna et al. 2009).  Several studies have also shown that

the expression of ppOX mRNA (Ida, Nakahara et al. 2000) and orexin receptors

(Heydendael, Sharma et al. 2011) are upregulated in rats pre-exposed to stressful

conditions.  In addition, research has shown that administration of orexin peptides into

the brain produces increased stress-like behaviors including freezing, grooming and

avoidance (Li, Li et al. 2009; Li, Li et al. 2010; Li, Li et al. 2010).  The role of orexins in

stress has also been shown in several studies that have demonstrated that orexins

promotes the physiological responses to stressful situations including increases in heart

rate and blood pressure (Kayaba, Nakamura et al. 2003; Zhang, Sakurai et al. 2006;

Furlong, Vianna et al. 2009).  Orexins are also involved in the elevation of HPA axis

function during repeated swim stress (Heydendael, Sharma et al. 2011).  In conclusion,

emerging evidence supports the view that orexin neurons are activated under a variety of

stressful circumstances and orexins regulate the stress-like physiological and behavioral

responses.

Harris et al. have proposed that there is a functional dichotomy between different

population of orexin neurons.  Specifically, orexin neurons located in the lateral

hypothalamus are associated with rewarding process, while those located in the medial

hypothalamic area (including perifornical and dorsomedial hypothalamic area) are

involved in arousal and stress (Harris, Wimmer et al. 2005; Harris and Aston-Jones 2006).

In a conditioned place-preference model, rats that were conditioned to a drug or food

reward showed a greater cFos expression in the orexin neuron found in the lateral but not

in the medial population (Harris, Wimmer et al. 2005).  Studies have also reported that

more orexin neurons in the medial hypothalamus show cFos expression during the active

period. In contrast, orexin neurons in the lateral hypothalamus do not appear to display diurnal variation (Estabrooke, McCarthy et al. 2001). In addition, the medial but not the lateral population of orexin neurons is activated during stress-induced reinstatement of drug-seeking behavior (Lu, Shepard et al. 2003), indicating that the stressors preferentially activate the medial population. These studies suggest that subgroups of orexin neurons may be responsible for different functions and that the medial population is likely to be involved in functions associated with arousal and stress.

It has been shown that stress activates orexin neurons through a CRF-mediated mechanism. CRF in the paraventricular nucleus of hypothalamus (PVN) was initially identified as the hypothalamic factor responsible for stimulating ACTH from the anterior pituitary gland (Owens and Nemeroff 1991). Anatomical evidence has shown that CRF-immunoreactive terminals make direct contact with orexin neurons and that orexin neurons express CRF receptors (CRF receptor-1 and CRF receptor-2) (Winsky-Sommerer, Yamanaka et al. 2004). It has been demonstrated that CRF can depolarize and activate orexin neurons in the brain slices via CRF receptor-1 mediated mechanism (Winsky-Sommerer, Yamanaka et al. 2004). Additionally, CRF receptor-1 knock-out mice showed an impaired activation of orexin-expressing neurons during footshock and restraint stress (Winsky-Sommerer, Yamanaka et al. 2004). On the other hand, CRF neurons appear to be regulated by orexins neurons. For example, central injection of orexin peptide increased the activity of CRF neurons (Sakamoto, Yamada et al. 2004) as well as the expression of CRF mRNA (Al-Barazanji, Wilson et al. 2001), whereas blockade of orexin receptors before the exposure to stress prevented the CRF mRNA elevation (Heydendael, Sharma et al. 2011). Additionally, central administrations of CRF

receptor antagonists attenuated the stress-like effects produced by microinjections of orexin-A peptide (Li, Li et al. 2010). The reciprocal circuit between CRF and the orexin system may contribute to the physical and behavioral responses to stress. Taken together, evidence from multiple sources indicates that a CRF-mediated activation of the orexin system may contribute to the high levels of arousal produced by a stressful situation and may mediate some of the behavioral responses associated with acute stress.

### 1.3.4. Orexins in the Regulation of Fear and Anxiety

Anatomical studies show that orexin neurons project to the brain regions associated with emotional processing of fear and anxiety, such as the amygdala (Bisetti, Cvetkovic et al. 2006) and the BNST (Schmitt, Usunoff et al. 2012). The amygdala has been shown to play an important role in associating a stimulus with an aversive event (fear learning) and the expression of fear (Davis 1998; Walker and Davis 2002; Davis, Walker et al. 2010), while the BNST appears to be involved in mediating anxiety (Davis 1998; Walker, Toufexis et al. 2003; Davis, Walker et al. 2010). Moreover, orexin neurons receive inputs from the amygdala and BNST which places the orexin neurons in a unique position to coordinate the physiological and behavioral responses to fear and anxiety (Sakurai, Nagata et al. 2005; Yoshida, McCormack et al. 2006).

Studies have shown that orexins are involved in the physiological and behavioral components of fear. For example, the dual orexin receptor antagonist almorexant can block the sympathetic component of the cardiac response (increased heart rate and

arterial pressure) of contextual fear (Furlong, Vianna et al. 2009; Zhang, Zhang et al. 2009).  Another study showed that orexin receptor antagonism attenuated fear-potentiated startle response (Steiner, Lecourt et al. 2012), a behavioral response used to quantify fear (Brown, Kalish et al. 1951; Davis 1992).  Interestingly, narcoleptic patients with deficits in the orexin system do not exhibit startle responses to unpleasant stimuli (Khatami, Birkmann et al. 2007; Ponz, Khatami et al. 2010), which suggests that blocking of the orexin signals attenuates fear.  However, the neural mechanisms by which orexins contribute to fear are not known at the present.  It is possible that orexin neurons enhance the heightened levels of arousal that promote fear expression and as well as the physiological and behavioral changes associated with fear.

A number of studies also indicate that orexins play a role in anxiety.  For example, microinjections of orexins in the cerebral ventricles, midline thalamus, and the BNST have been reported to produce anxiety and avoidance-like behaviors in the open field, social interaction, light-dark exploration and elevated plus-maze tests (Suzuki, Beuckmann et al. 2005; Li, Li et al. 2009; Li, Li et al. 2010; Li, Li et al. 2010; Heydendael, Sharma et al. 2011; Lungwitz, Molosh et al. 2012).  Additionally, recent studies have reported that orexins help to mobilize the coordinated behavioral and physiological responses associated with panic attacks (Johnson, Molosh et al. 2012; Johnson, Samuels et al. 2012).

As discussed earlier, exposure of rodents to brief inescapable footshocks presents a reliable mean of creating a PTSD-like condition in this species (van Dijken, Mos et al. 1992; Louvart, Maccari et al. 2005; Siegmund and Wotjak 2007; Mikics, Baranyi et al. 2008).  Previous work in our laboratory has provided evidence that supports a role for

orexins at the level of the midline thalamus in the regulation of fear and anxiety (Li, Li et al. 2009; Li, Li et al. 2010; Li, Li et al. 2010). One particularly important finding was that microinjection of an $OX_2$ receptor antagonist in the midline thalamus was found to produce anxiolytic effect in rats that were previously exposed to a single 10s episode of 2.0 mA footshocks (Li, Li et al. 2010). These results point to the possibility that exposure to a fearful or stressful situation upregulates the orexin system in a way to promote fear and anxiety. The experiments described in this thesis examine this general hypothesis.

## 1.4. Objectives

This thesis presents experiments that were designed to examine the role of orexins in a rat model of PTSD. First of all, experiments were done to characterize and further validate the footshock model of PTSD. This was followed by a series of experiments which examined whether the activity of orexin neurons was increased following exposure of rats to footshocks and if these increases were due to activation of CRF receptors at the time of the footshocks. Finally, a series of pharmacological experiments were done to determine whether systemic injection of a nonselective orexin receptor antagonist decreases the expression of fear and anxiety in rats following exposure to a single episode of footshocks.

## 1.5. Hypotheses

### 1.5.1. Hypothesis 1: Early Fear Response Predicts Anxiety

People who experienced traumatic events usually initially have some of the symptoms associated with PTSD but these symptoms eventually normalize. The fact that the majority of traumatized individuals do not go on to develop PTSD underscores the importance of individual differences in the recovery from a life threatening experience (Kessler, Sonnega et al. 1995; Breslau and Kessler 2001). While it has been difficult to establish a causal link between the early effect of trauma (peritraumatic period) and the subsequent development of PTSD in humans (Harvey and Bryant 2002; Shalev 2002), it is possible that differences in how individuals respond immediately after the trauma may determine whether a person develops PTSD (Cardinal, Parkinson et al. 2002). Part of the difficulty in providing clinical evidence for such a link is that studies which have examined this question have to use heterogeneous populations of subjects exposed to traumatizing events with varying levels of intensity. The advantages of using a rat model to determine whether early fear is related to the long-term expression of PTSD-like behaviors is provided by the fact that the fear-inducing conditions can be well-controlled and that the subjects are from a relatively homogenous population. **The hypothesis that the acute generalized fear response in rats that previously received moderately intense footshocks predicts the long-term expression of anxiety was tested.** Shock rats were separated into high responders (HR) and low responders (LR) based on the amount of immobility expressed to a novel tone one day after the shock exposure.

Avoidance behavior was examined in the social interaction and defensive withdrawal tests at 14 or 30 days after the shock exposure.

### 1.5.2. Hypothesis 2: Extinction of Fear to the Shock Chamber Attenuates Novelty Fear

Studies have shown that rats that received footshocks display fear to a novel test chamber and a novel tone (Van Dijken, Van der Heyden et al. 1992; Bruijnzeel, Stam et al. 2001; Louvart, Maccari et al. 2005). The fear response to a novel chamber and tone may be due to associative learning mechanism (fear generalization) or non-associative learning mechanism (fear sensitization) (Kamprath and Wotjak 2004; Daviu, Fuentes et al. 2010). **The hypothesis that the fear to novelty is due to a combination of fear generalization and fear sensitization mechanism was tested.** To test this hypothesis, experiments were done to examine whether the fear responses to a novel chamber and a novel tone are attenuated in rats re-exposed to the shock chamber until the associative fear (fear to the shock context) was eliminated.

### 1.5.3. Hypothesis 3: Footshocks Produce a Lasting Upregulation of ppOX Synthesis

Orexins are known for their roles in supporting arousal and previous studies have shown that orexin neurons are activated after an acute stress episode including footshocks (Ida, Nakahara et al. 2000; Zhu, Onaka et al. 2002; Espana, Valentino et al. 2003; Winsky-Sommerer, Yamanaka et al. 2004; Furlong, Vianna et al. 2009). However, it is

not known whether there is a lasting change in the activity of orexin neurons after stressful experience.  As mentioned earlier, rats that received a single episode of footshocks expressed fear and anxiety which can last for at least one month (Louvart, Maccari et al. 2005; Mikics, Toth et al. 2008).  **The hypothesis that the footshock stress produces a long-term upregulation in ppOX synthesis was tested.**  Experiments were done to determine whether the synthesis of the ppOX mRNA is increased in rats exposed to relatively intense footshocks (1.5 mA).  The relationships between ppOX mRNA levels and immobility responses to a novel chamber (generalized fear) or the shock chamber (contextual fear) were also examined.

### 1.5.4.  Hypothesis 4: More Orexin Neurons are Activated in Shock Rats Placed in Situations Involving Fear.

Several studies have shown that orexin neurons become activated when rats were exposed to a acute stressful condition (Zhu, Onaka et al. 2002; Espana, Valentino et al. 2003; Furlong, Vianna et al. 2009).  It is not known whether the activity of orexin neuron is increased more strongly in shock rats exposed to a novel environment or the shock context.  **The hypothesis that the activity of orexin neurons is enhanced in shock rats placed in conditions that promote fear was tested.**  The experiments aimed to verify the activity of orexin neurons.  A double labeling procedure staining for cFos protein and orexin neurons was used to label and quantify the number of activated orexin neurons in shock and nonshock rats placed in a novel chamber (generalized fear) or the shock chamber (contextual fear) at 14 days after the shock exposure

### 1.5.5. Hypothesis 5: Blocking of CRF Receptor-1 Attenuates ppOX mRNA Synthesis and Fear Expression in Rats that Received Footshocks

CRF is believed to modulate behaviors associated with fear and anxiety (Kikusui, Takeuchi et al. 2000; Zorrilla, Valdez et al. 2002; Bijlsma, van Leeuwen et al. 2011). A recent study has demonstrated that orexin neurons are activated by CRF and produce stress-like behavior (Winsky-Sommerer, Yamanaka et al. 2004). When animals were exposed to footshocks, activation of CRF receptor-1 may produce a lasting change in orexin neuron activity. **The hypothesis that blockade of CRF receptor-1 at the time of the footshock exposure produces a lasting decrease in orexin neuron activity and fear-like behaviors was tested.** Experiments were done to see if blocking the CRF receptor-1 using antalarmin reduced fear to a novel chamber and the shock context during a two week period. The levels of ppOX mRNA was quantified after the behavioral tests. The relationships between ppOX RNA levels and immobility responses to the novel or shock chamber were also analyzed.

### 1.5.6. Hypothesis 6: Blocking of Orexin Receptors Attenuates Fear And Anxiety

Orexin are also involved in fear and anxiety. Previous studies have shown that blocking of orexin receptors can reduce the cardiac response of contextual fear (Furlong, Vianna et al. 2009), and fear-potentiated startle response (Steiner, Lecourt et al. 2012). Blocking of orexin receptors also decreases the behavioral and physiological response associated with panic attacks (Johnson, Molosh et al. 2012; Johnson, Samuels et al. 2012)

and anxiety (Li, Li et al. 2010). **The hypothesis that an orexin antagonism attenuates long-lasting expression of fear and anxiety in rats that received the footshocks exposure was tested.** Systemic injection of the nonspecific orexin receptor antagonist TCS-1102 were done before placing shock and nonshock rats in a big open field or in the shock chamber at 14 days after the shock exposure.

### 1.5.7. Hypothesis 7: Blocking of Orexin Receptors Attenuates Anxiety in a Subgroup of Shock Rats with High Levels of Anxiety

Previous studies have shown that individual differences in the acute fear responses to a novel tone account for the variability in anxiety-like behaviors (avoidance) displayed long time after shock procedure (Rau, DeCola et al. 2005; Chen, Li et al. 2012). Accordingly, shock rats that are grouped as high responders (HR) based on the amount of immobility expressed to a novel tone one day after the shock exposure display high levels of anxiety for several weeks after the shock exposure. **The hypothesis that systemic injection of an orexin receptor antagonist has anxiolytic effects only in a subgroup of shock rats with high levels of anxiety was tested.** Groups of nonshock, LR, and HR rats were tested with systemic injections of a nonselective dual orexin receptor antagonist TCS-1102 were exposed to the ETM 14 days after the shock exposure to test their avoidance and escape behaviors.

# Chapter 2

# Materials and Methods

## 2.1. Animals

Experimental subjects were adult male Sprague-Dawley rats weighing $140 \pm 20$ g upon arrival. They were pair-housed and kept in plastic cages in a colony room on a 12-hour light-dark cycle (lights on 06:00 am) with controlled temperature (20 - 24 ℃) and humidity (40 - 70 %). Animals had free access to food, water and rodent retreat shading in their home cages. All of the experimental procedures were in compliance with the Canadian Council on Animal Care and were approved by Protocol Management Review Committee of University of Manitoba.

## 2.2. Handling and Testing

Animals were handled for 5 min on alternate days during a 10-day adaption period before the footshock procedure and behavioral tests. All behavioral procedures were done in the light cycle of the day (08:00 - 16:00). Animals were transferred one at a time from

their colony room to footshock room A for footshock delivery, and to behavioral test room B for behavioral tests. After exposure of each rat to test chamber, the shock chamber was cleaned with alcohol (10%) and the behavioral test chambers were cleaned with liquinox (0.5%) and dried in order to prevent interference of subsequent tests by olfactory cues. All the behaviors were videotaped and subsequently scored by two experimenters blind to the experimental conditions. The reliability score between raters (correlation coefficient) ranged between 0.94 - 0.97 and the data from two observers were averaged for statistical analysis.

## 2.3. Footshock Procedure

Rats were transferred one at a time to a brightly illuminated room (400 - 500 lx) dedicated exclusively for the delivery of footshocks. After a 2 min acclimation period in a commercially available chamber (MED Associates, St. Albans, Vermont, USA), rats received 5 electric footshocks (1.5 mA, 2 s, the interval between shocks ranged from 10 - 50 s randomly presented over 2 min) through a grid floor of the chamber. The rats (referred to as "shock rats") were kept in the chamber for another 60 s before they were returned to their home cages. The rats from the nonshock group were placed in the shock chamber for the same amount of time but received no footshocks. The shock chamber was cleaned and the bedding under the grid floor was changed after exposure to each rat.

## 2.4.  A Rat Model of PTSD

### 2.4.1.  Acute Fear Response to Novel Environment

This experiment examined whether the intensity of the acute fear response can predict the long-term expression of anxiety in shock rats.  The timeline of the footshock exposure and the behavioral tests is shown on Table 1.  Rats received 5 footshocks in footshock room A as described before whereas nonshock rats were placed at the same chamber for equal time but no shock was delivered.  One day after the shock exposure, rats were transferred to behavioral test room B and placed in a novel chamber (made of black Plexiglas and measuring L65 cm $\times$ W40 cm $\times$ H50 cm) with a light intensity of 3 - 5 lx.  The duration of the test was 6 min which was composed of a 3 min period with background noise (60 dB) and the second 3 min period with a novel auditory tone presented (9 kHz, 75 dB).  Their acute post-shock fear response was assessed by measuring the amount of immobility percentage (the immobility duration / total exposure time) for both test conditions.  Immobility was defined as completely lack of body movement except breathing movements as previously described (Fanselow 1980).

### 2.4.2.  Fear Response to Novel Chambers and the Shock Chamber

The fear responses to novel chambers were assessed on Days 11 and 26 after the shock exposure.  On Day 11, rats were placed in a novel clear plastic chamber (L22 cm $\times$ W28 cm $\times$ H35 cm) for 5 min.  On Day 26, rats were placed in the center of

**Table 1  Timeline of the behavioral tests in a rat model of PTSD**

| | |
|---|---|
| Day 0 | Shock exposure |
| Day 1 | Small open field and novel tone test |
| Day 10 | Social approach and avoidance test |
| Day 11 | Novel plastic box test |
| Day 12 | Shock chamber exposure |
| Day 25 | Social approach and avoidance test |
| Day 26 | Big open field test |
| Day 27 | Shock chamber exposure |
| Day 31 | Defensive withdrawal test |

a large open field (L80 cm $\times$ W80 cm $\times$ H40 cm) for 5 min. Both chambers were

illuminated with a light of 8 - 12 lx and the amount of immobility displayed by the rats

was calculated for both novel conditions. In addition, ultrasonic vocalizations (USV)

emitted during the novel chamber exposure on Day 11 were recorded using a specialized

detector (Med Associates), and the recording was later analyzed for the presence of calls

within the dysphoric range (20-30 kHz). The contextual fear response was measured on

Days 9 and 27 by placing the rats in the same shock context for 5 min (no shock

delivered). The amount of immobility and the number of USV emitted in the shock

chamber were recorded and analyzed as described above.


### 2.4.3. Social Approach and Avoidance Test


Social anxiety was examined in a three-compartment black Plexiglas chamber

(L65 cm $\times$ W40 cm $\times$ H50 cm) on Days 10 and 25. The chamber contained three parts:

one compartment with mesh wall facing the interaction zone (L15 cm $\times$ W40 cm $\times$ H50

cm, compartment A), interaction zone (L30 cm $\times$ W40 cm $\times$ H50 cm) and a compartment

with a sliding door (10 cm $\times$ 10 cm) on the wall facing the interaction zone (L20 cm $\times$

W40 cm $\times$ H50 cm, compartment B). The test chamber was placed in a dimly lit testing

room (3 - 5 lx) and the test procedure was performed as described before (Haller, Leveleki

et al. 2003). Briefly, the animal was placed into compartment B and an unfamiliar rat was

placed in compartment A. After a 3 min habituation period, the door between

compartment B and the interaction zone was open allowing the test rat to explore the

chamber for 5 min. While the rats can smell and see each other, the mesh wall between

the compartment A and interaction zone prevents the rats from having actual physical contact. The latency to enter the social interaction zone for each rat was recorded for further analysis.

### 2.4.4. Defensive Withdrawal Test

The defensive withdrawal test was done on Day 31 using an open field (L80 cm × W80 cm ×H40 cm) with a black floor which was illuminated at 8 - 10 lx. The test protocol was similar to what was described in previous studies (Gutman, Owens et al. 2003; White, Kalinichev et al. 2007). The rat was placed into a black withdrawal chamber (L25 cm × W20 cm ×H15 cm). Then the withdrawal chamber was placed along a wall about 20 cm from one of the corners of the open field. Two minutes later, the sliding door from side of the withdrawal chamber was removed to allow the rat to freely explore the open field for 10 min. The latency to enter the open field (four paws into the open arena) was quantified by observers blind to the experimental design.

### 2.4.5. Statistical Analyses

The immobility displayed in a novel chamber and in the presence of the tone one day after the shock exposure were analyzed using two-tailed Student's t-test to compare the differences between nonshock and shocked groups. Shocked rats were subdivided into low responders (LR) or high responders (HR) based on the amount of immobility

expressed during the presentation of the novel tone (see Results for more information on the criteria used). Group differences between nonshock, LR and HR rats were assessed by Kruskal-Wallis one-way analysis of variance by ranks followed by a multiple comparison non-parametric adapted t-test when appropriate ($p < 0.05$). A value of $p < 0.05$ was considered to be significant and the data were presented as mean $\pm$ SEM. Statistical analysis was done by SPSS 19.0 software.

## 2.5. Fear Extinction Experiment

### 2.5.1. Fear Extinction Training

While there is some evidence that the expression of fear to novel chambers in shock rodents is a fear generalization process (Golub, Mauch et al. 2009; Sauerhofer, Pamplona et al. 2012), other evidence supports the view that this also involved fear sensitization mechanisms (Siegmund and Wotjak 2007). The experiments described here were done to examine whether extinction of fear in shock rats decreases the generalized fear displayed by these rats when they are placed in a novel chamber and when exposed to a novel tone. The timeline of the proposed experiment is shown on Table 2. Rats were placed in the shock chamber and received footshocks and nonshock rats were placed in the same chamber for equal time but no shock was delivered. One day after

**Table 2  Timeline of the behavioral tests in fear extinction experiment**

| | |
|---|---|
| Day 0 | Shock exposure |
| Day 1 | Shock chamber exposure |
| Day 7-12 | Fear extinction training |
| Day 13 | Shock chamber exposure |
| Day 15 | Big open field test |

the shock exposure, rats were transferred to behavioral test room B and exposed to the shock chamber for 5 min. The percentage of immobility was recorded, and was used for separation of shock rats into two homogenous groups with equal immobility response. The timing of the extinction training is important for reduction of fear and anxiety. Previous study has shown that early fear extinction (one day after the shock) enhances acoustic startle response in stressful rats (Golub, Mauch et al. 2009). In the present experiment, fear extinction training started one week after the shock exposure.

During the period of extinction, shock and nonshock rats were placed in the shock chamber for 15 min each day over 4 consecutive days until extinction of fear was complete. Another group of shock rats was placed in a new home cage for the same amount of time to control for extinction (shock/control). The amount of immobility displayed during the first 5 min of the chamber exposures was calculated. After fear extinction was complete, rats were placed in the shock chamber on Day 13 to test the effect of the extinction.

## 2.5.2. Effects of Extinction on Fear Response to Novel Environment

The novelty test was performed at Day 15. Rats were placed for 6 min in a big open field (L80 cm $\times$ W80 cm $\times$ H40 cm, with the floor of the chamber marked off as 49 rectangles of L11 cm $\times$ W12 cm) which was illuminated by a light of 8 - 12 lx, and a tone (9Hz, 75dB) was presented during the last 3 min of the test. Percentage of immobility and

locomotor activity (number of floor lines crossed by the rat) were calculated for both test session.

### 2.5.3. Statistical Analyses

A one-way ANOVA was used to analyze the immobility and locomotor activity between nonshock, shock/control, and shock/extinction groups. Fisher's Least Significant Difference (LSD) post-hoc analysis was used when significant effects were observed. Two-tailed Student's t-test was applied to compare the immobility between shock/extinction and nonshock groups during extinction training. A value of $p < 0.05$ was considered to be significant and the data were presented as mean $\pm$ SEM. Data analyses were performed using SPSS software, version 19.0.

## 2.6. Effects of Footshocks on ppOX mRNA Levels

### 2.6.1. Behavioral Procedure

The aim of these experiments was to evaluate ppOX mRNA levels in shock and nonshock rats at 6 and 14 days post-shock. The timeline of the footshock exposure and the behavioral tests is shown on Table 3. Animals were placed in the shock chamber and received footshocks at Day 0, while the control group was exposed to

**Table 3  Timeline of the behavioral tests in ppOX mRNA levels experiments**

| *Group1* | *Levels of ppOX mRNA at 6 days post-shock* |
|---|---|
| Day 0 | Shock exposure |
| Day 1 | Small open field test |
| Day 2 | Shock chamber exposure |
| Day 6 | Brains processed for in situ hybridization |

| *Group 2* | *Levels of ppOX mRNA at 14 days post-shock* |
|---|---|
| Day 0 | Shock exposure |
| Day 9 | Small open field test |
| Day 10 | Shock chamber exposure |
| Day 14 | Brains processed for in situ hybridization |

the same shock chamber but footshocks were not given.  Rats that were sacrificed at 6 days after the footshock exposure were transferred one by one to behavioral test room B, and placed in a small open field (L 65 cm × W 40 cm × H 50 cm, black Plexiglass wall and floor; light intensity of 3 - 5 lx) for 5 min on Day 1 to assess fear response to a novel chamber (generalized fear response).  On the following day (Day 2), the rats were placed in the shock chamber (light intensity of 400 - 500 lx; no shock delivered) for 5 min to evaluate fear response to the shock context (contextual fear response).  The percentages of immobility displayed were scored for both tests.  Another group of rats that were sacrificed 14 days post-shock was also placed in a small open field (L 65 cm × W 40 cm × H 50 cm, black Plexiglass wall and floor; light intensity of 3 - 5 lx) for 5 min on Day 9, and in the shock chamber (light intensity of 400 - 500 lx; no shock delivered) for 5 min on Day 10 to evaluate the generalized and contextual fear responses.  The percentages of immobility were scored for both test days.  The rats were anaesthetized and perfused, and their brains were collected for processing in situ hybridization.

## 2.6.2.  In Situ Hybridization

The rats were anaesthetized with chloral hydrate (600 mg/kg, i.p.) and perfused transcardially with 200 ml ice-cold 0.1M phosphate buffered saline (PBS; pH 7.4) solution.  The brains were rapidly removed and frozen with dry ice and stored at -80 ℃ for sectioning on a later date.  Coronal 20 μm sections of the posterior hypothalamus were made using a cryostat and mounted on slides that were subsequently stored at -80 ℃ until the hybridization procedure was done.

All hybridization solutions were treated with 1% diethylpyrocarbonate and autoclaved to inhibit RNase activity. Frozen brain sections were thawed and fixed with ice-cold 4% paraformaldehyde (pH 7.4) for 10 min followed by a rinse in 0.1 M PBS solution for 10 min. The sections were acetylated in 0.1 M triethanolamine (pH 8.0) containing 0.25% acetic anhydride for 10 min, washed with $2 \times$ sodium chloride-sodium phosphate-EDTA buffer ($2 \times$ SSC; 0.3 M NaCl, 0.03 M sodium citrate, pH 7.0) for 10 min, and dehydrated with 50%, 70%, 95% and 100% ethanol (1 min each). Subsequently, the sections were delipidated in chloroform solution for 8 min and washed with 100% ethanol followed by rehydration in 95% and 80% ethanol (1 min each).

A 36-mer synthetic oligonucleotide probe (TTC GTA GAG ACG GCA GGA ACA CGT CTT CTG GCG ACA; complementary to bases 115 - 150 of rat ppOX) (Yamamoto, Ueta et al. 1999) was labeled at the 3'-end with $^{32}$P-dATP (BLU512A250UC, Perkin Elmer, Waltham, MA) to a specific activity of > 12 TBq/mmol by using terminal deoxynucleotideyl transferase (Cat. # M1871, Promega, Madison, WI). The probe for ppOX mRNA does not display homology to any other known sequences as shown using homology screening of Genebank/EMBL sequences. The labeling reactions were done in a 37 ℃ water bath for 90 min and the radio-labeled probe was purified using Illustra Probe Quant G-50 Micro Columns (28-9034-08, GE Healthcare, Buckinghamshire, UK). The purified probe was mixed with Ultrahyb-oligo hybridization buffer (AM8663, Applied Biosystems, Frederick, MD), heated to 70 ℃ for 10 min, and then cooled on ice. The final concentration of the probe in the hybridization buffer was in a range of 1.8 - 2.2 pmol/ml and had a specific activity of $10^7$ cpm/ml.

Hybridization was done by covering the sections with 100 μl of hybridization mixture and incubating them overnight at 38 ℃. The negative control experiments were done by adding 100-fold excess of the unlabeled probes to the sections. The slides were rinsed in $1 \times$ SSC and post-washed four times in 50% formamide in $2 \times$ SSC solutions for 30 min at room temperature followed by post-wash in $1 \times$ SSC solution for one hour repeated 3 times. After dehydration with graded ethanol (50%, 70%, 90%, 100%, 1 min each), the slides along with autoradiographic $^{14}$C microscale standard strip (31 - 883 nCi/g) were exposed to Amersham hyperfilm (GE Healthcare, Buckinghamshire, UK) for 12 days in cassettes. The hyperfilms were developed with Kodak GBX developer and fixer (Sigma-Aldrich, St. Louis, MO).

### 2.6.3. Imaging

Autoradiographic images of individual sections with the densest ppOX mRNA labeling (Bregma -3.0 to - 3.3) were photographed with Olympus BX51microscope equipped with a digital camera (SPOT RT Slicer, Diagnostic Instruments Inc, Sterling Heights, MI). Each part for individual section was sampled manually by using the selection tool to make a rectangular (as shown in Figure 1a) selection area bilaterally on the region of interest and analysed by NIH Image-J software. The medial and lateral parts of orexin population (as shown in Figure 1a,b) were separated by a line drawn vertical besides medial fornix. The optical density of the areas of the film not involving the orexin population was subtracted from the optical density of the orexin population.

**Figure 1  Photomicrograph of the lateral hypothalamus showing orexin neurons**

Images show rat coronal hypothalamus sections.  (a) Immunohistochemistry labeling with orexin neurons.  The area surrounded by the rectangular includes all orexin neurons, and this area will be used for quantification of ppOX mRNA.  Orexin neurons distributed medially to the fornix (shown as right to the drawn line) are referred as medial population of orexin neurons, and the other part is referred as lateral population of orexin neurons. (b) In situ hybridization labeling of ppOX mRNA showing the medial and lateral population of orexin neurons.  M: medial; L: lateral; F: Fornix.

a



Bregma -3.2

F

L          M

b



F

L          M
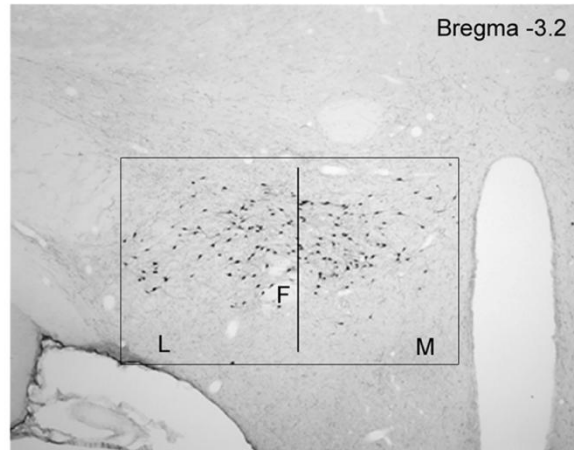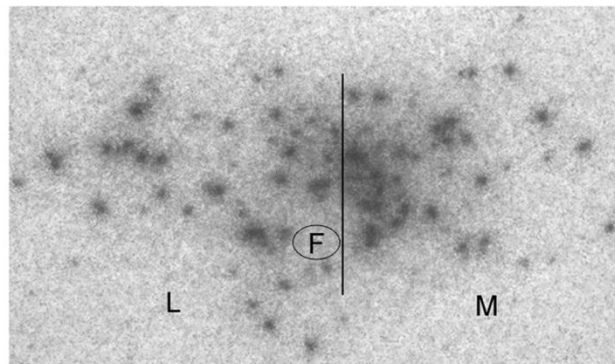
The mean optical density of each region, which was used for statistical analysis, was interpolated along the calibration curve derived from the optical density of $^{14}$C microscale standards.

### 2.6.4. Statistical Analyses

The results of the behavioral tests and ppOX mRNA level data were analyzed using two-tailed Student's t-test. Pearson Correlation was applied to examine the relationship between the total ppOX mRNA levels and immobility percentage to the novel and shock chambers. A value of $p < 0.05$ was considered to be significant and the data were presented as mean $\pm$ SEM. Data analyses were performed using SPSS software, version 19.0.

## 2.7. Changes in the Activity of Orexin Neurons During Expression of Fear

### 2.7.1. Behavioral Procedure

The aim of this experiment was to determine whether orexin neurons are activated in rats that are exposed to either a novel chamber or the shock chamber at 14 days after the exposure to footshocks. The cFos protein was used as an anatomical marker of

excitation of orexin neurons (double-labeling protocol). The timeline of the procedures is shown on Table 4. Rats were placed in the shock chamber and received electric footshocks, whereas nonshock rats were exposed to the same chamber but no shock was given at Day 0. Studies have shown that individual differences in the acute reaction to a novel tone after the footshock exposure account for the variability in expression of anxiety-like behaviors displayed later by shock rats (Rau, DeCola et al. 2005; Chen, Li et al. 2012). One day after the shock exposure, rats were exposed to a small open field for 6 min (L 65 cm $\times$ W 40 cm $\times$ H 50 cm, black Plexiglass walls and floor, illuminated by lights of 3 - 5 lx) and a novel auditory tone (9 kHz, 75 dB) was presented during the last 3 min of exposure. The immobility expressed in response to the novel tone was used to generate homogenous subgroups of rats. Fourteen days after the initial shock exposure, three subgroups of rats with similar levels of immobility to the novel tone were placed for 15 min in one of the following test chambers: a clean home cage containing a small shading compartment, a clear cylindrical chamber (28 cm diameter and illustrated at 8 - 12 lx), or the shock chamber (bright lights of 400 - 500 lx). Videotapes of the rats' behavior during the first 5 min were analyzed to compare the immobility and activity levels between nonshock and shock rats tested in the different chambers. The percentage of duration of staying in shading (the hiding duration/total exposure time) and the duration of rearing (standing on hind legs) were quantified for the group exposed to home cage. The percentage of immobility, and the duration of rearing were calculated for the rats placed in the novel chamber or the shock chamber. Rats were deeply anaesthetized and perfused 90 min after the exposure and their brains were collected for immunohistochemical staining.

**Table 4  Timeline of the behavioral tests in orexin neuron activity experiment**

| Day 0 | Shock exposure |
|---|---|
| Day 1 | Small open field and novel tone test |
| Day 14 | Exposure to one of the conditions: home cage, cylindrical chamber, shock chamber; brains processed for immunohistochemistry |

## 2.7.2. Immunohistochemistry

Animals were anaesthetized with chloral hydrate (600 mg/kg, i.p.), and then immediately perfused transcardially with 150 ml 0.9% saline solution with 0.5% heparin, followed by 500 ml ice-cold fixative (4% paraformaldehyde in 0. 1 M PB solution). The brains were removed and post-fixed in the same fixative for 60 min at 4 ℃, stored at 20% sucrose PBS solution for 2 days, then transferred to 30% sucrose PBS solution. Coronal sections of the brain were cut at 50 μm in a cryostat and placed in 0.1 M PBS solution with 0.1% sodium azide and stored at -20 ℃ in an ethylene glycol cryoprotectant solution until further processing.

A double-labeling protocol was used to label orexin neurons and neurons expressing cFos protein. All immunohistochemical staining was carried out on free-floating sections at room temperature. Frozen sections were thawed and rinsed in 0.1 M PBS solution for 3 times (10 min each). The same rinsing procedure was applied after each reaction and antibody incubation. Sections were oxidized with 1% hydrogen peroxidase PBS solution for 10 min, and then pre-incubated in the blocking solution (5% normal donkey serum, 0.1% sodium azide, and 0.3% Triton X-100 in PBS solution) for 60 min. Primary and secondary antibodies were diluted in the same blocking solution. Sections were then incubated in a primary antibody rabbit anti-cFos (1:10,000; Calbiochem, Merck KGaA, Darmstadt, Germany) for 20 hrs. Subsequently, sections were transferred to a secondary antibody of biotinylated donkey anti-rabbit (1:500; Jackson Immunoresearch, West Grove, PA) for 2 hrs, followed by exposing in an avidin-biotin complex (1:500; Elite ABC Kit; Vector Laboratories, Burlingame, CA) for 60 min. After

a few more rinses, the tissue was reacted for 8 min with diaminobenzidine (DAB) with nickel intensification (Vector DAB Kit) to produce dark cFos protein labeling in cell nucleus. The DAB reaction was terminated by rinsing sections in 0.1 M PBS solution. Afterward, sections were incubated in another primary antibody goat anti-orexin B (1:500; Santa Cruz Biotechnology, Inc, Santa Cruz, CA ) for 20 hrs, followed by incubating with biotinylated donkey anti-goat antiserum (1:500; Jackson Immunoresearch) for 2 hrs. Sections were then processed in ABC solution for 60 min and then reacted for 3 min in DAB without nickel intensification to produce brown orexin neuron labeling. At last, sections were then mounted onto gelatin-coated slides, air-dried and coverslipped with cover glass. Negative controls (leaving out the primary antibody) were used to establish the level of nonspecific staining.

### 2.7.3. Quantification of Fos-positive Orexin Neurons

Two sections with the most dense orexin-positive neurons (Bregma -3.0 to -3.3) of each animal were selected under the microscope, and counted for the total number orexin neurons staining and the number of orexin neurons expressing cFos protein (a section from each subgroup of shock rats was selected as shown examples in Figure 9, page??). The medial and lateral parts of the orexin population separated by an imaginary line drawn vertical besides medial fornix was used to demarcate and count the number of double labeled neurons. The results were expressed as the percentage of Fos-positive orexin neurons form the total number of single labeled orexin neurons.

### 2.7.4.  Statistical Analyses

Behavioral data when rats were exposed to home cage, novel or shock chamber were analyzed using two-tailed Student's t-test to compare differences displayed by nonshock and shock rats.  A two-way ANOVA was used to evaluate the main and interaction effect between "shock" (nonshock or shock) and "exposure place" (home cage, novel chamber, or shock chamber) on percentage of neurons double-labeled for cFos/orexin.  If the ANOVA revealed significant effects, a Student's t-test was used to evaluate the "shock" effect on cFos/orexin neurons by comparing the shock and nonshock groups which were placed to one of the exposure places.  A one-way ANOVA was performed to compare the effect of "exposure place" within nonshock and shock groups, and Fisher's LSD post-hoc test was used to compare the specific group differences if any significance was found.  A value of $p < 0.05$ was considered to be significant and the data were presented as mean $\pm$ SEM.  Data analyses were performed using SPSS software, version 19.0.

## 2.8. Effects of the CRF Receptor-1 Antagonist Antalarmin on Fear and ppOX mRNA Levels

### 2.8.1. Pharmacological Tests

This experiment was done to evaluate whether the CRF receptor-1 antagonist antalarmin attenuates the expression of contextual and generalized fear in rats pre-exposed to footshocks. Antalarmin hydrochloride (N-Butyl-N-ethyl-2,5,6-trimethyl-7-(2,4,6-trimethylphenyl)-7H-pyrrolo[2,3-d]pyrimidin-4-amine hydrochloride; Cat.# 2778; Tocris, Minneapolis, MN) was dissolved in a solution contain 10% ethanol and 10% Cremophor (Sigma-Aldrich, St. Louis, MO). The timeline of antalarmin injection and behavioral tests is shown as Table 5. Rats received vehicle or antalarmin in a dose of 20 mg/kg intraperitoneally (i.p. 4 ml/kg) 30 min before the footshock exposure. Rats were then placed in the shock chamber and received footshocks whereas nonshock rats were exposed to the same chamber for equal time but no shock was delivered. The percentage of immobility and the number of USV within the dysphoric range (20 - 30 kHz) emitted during the period of shock administration was recorded.

The generalized fear response to novelty was assessed at Days 1 and 9 after the shock exposure. On Day 1, rats were placed in a small open field for 6 min (L65 cm × W40 cm × H50 cm, the floor of the chamber marked off as 6 rectangles of L22 cm × W20 cm, and illuminated with a dim light intensity of 3 - 5 lx) with a tone present in the last 3 min period (9 Hz, 75 dB against the background noise level of 45dB). On Day 9,

**Table 5  Timeline of antalarmin injection and the behavioral tests**

| | |
|---|---|
| Day 0 | Antalarmin or Vehicle injection, followed by shock exposure |
| Day 1 | Small open field and novel tone test |
| Day 2 | Shock chamber exposure |
| Day 9 | Big open filed and novel tone test |
| Day 10 | Shock chamber exposure |
| Day 14 | Brains processed for in situ hybridization |

rats were placed in the center of a large open field (L80 cm $\times$ W80 cm $\times$ H40 cm, with the floor of the chamber marked off as 49 rectangles of L11 cm $\times$ W12 cm, and illuminated by a light of 8 -12 lx) for 6 min, and a novel tone (5 kHz, 75 dB) was presented during the last 3 min of the test. The percentage of immobility and locomotor activity (number of floor lines crossed by the rat) were recorded for each session (No Tone or Tone) on both days.

The contextual fear was evaluated in rats that were exposed to the shock chamber for 5 min at Days 2 and 10. The percentage of immobility and the number of USV were recorded. Four days after the last behavioral test, rats were anaesthetized and rapidly perfused with 200 ml ice-cold 0.1 M PBS, and their brains were removed for processing in situ hybridization as described in the previous section. The films with ppOX labeling were developed and radioisotope emission on the film was imaged and quantified as described before.

### 2.8.2. Statistical Analyses

A two-way ANOVA was performed to investigate the main and interaction effects of "shock" (nonshock or shock) and "antalarmin" (vehicle or antalarmin) on fear responses and ppOX mRNA levels. Whenever a significant effect was found, the pairwise comparisons using Student's t-test were made to evaluate the "shock" effect by comparing nonshock and shock rats with the same treatments, and the "antalarmin"

effect by making comparisons of vehicle and antalarmin in the groups of nonshock and shock rats. Pearson correlation was used to analyze the linear relationship between ppOX mRNA levels and the immobility responses expressed to the novel chamber on Day 9 and the shock context on Days 10 for each group. A value of $p < 0.05$ was considered to be significant and the data were presented as mean $\pm$ SEM. Data analyses were performed using SPSS software, version 19.0.

## 2.9. Effects of the Orexin Receptor Antagonist TCS-1102 on Fear and Anxiety

### 2.9.1. Pharmacological Tests

The aim of this experiment was to evaluate wheater blocking of orexin receptors attenuates the expression of fear and anxiety in rats at 14 days post-shock. A dual orexin receptor antagonist N-biphenyl-2-yl-1-{[(1-methyl-1H-benzimidazol-2-yl)sulfanyl]acetyl}-L-prolinamide (Tocris,TCS-1102, cat# 3818; Minneapolis, MN) was used for this study. TCS-1102 blocks both $OX_1$ and $OX_2$ receptors and has been shown to be effective in blocking orexin-mediated behaviors (Bergman, Roecker et al. 2008; Winrow, Tanis et al. 2010). The timeline of the footshock exposure and the behavioral tests is shown on Table 6. Rats received footshocks at Day 0, and nonshock rats were placed in the shock chamber but no shock was given. One day after the footshock exposure, rats were placed in a small open field for 6 min (L 65 cm $\times$ W 40 cm $\times$ H 50 cm,

black Plexiglass walls and floor; light intensity of 3 - 5 lx) and a novel tone was presented during the last 3 min (9 kHz at 80 dB). Fear responses (immobility percentage) to the novel tone were used to generate homogenous subgroups for drug treatment.

Two weeks after exposure to the footshocks, TCS-1102 was freshly dissolved in the vehicle poly-ethylene glycol 200 (PEG200, Sigma Aldrich, Canada) as previously reported (Winrow, Tanis et al. 2010). The rats were injected intraperitoneally with either the vehicle or one of three different doses of TCS-1102 (5, 10, or 20 mg/kg) 30 min before behavioral tests. These doses were chosen based on their effectiveness in reducing motivational behaviors (Winrow, Tanis et al. 2010). The behavioral tests includes placing rats in an open field made of black Plexiglas (L 80 cm $\times$ W 80 cm $\times$ H 40 cm, with the floor of the chamber marked off as 49 rectangles of L11 cm $\times$ W12 cm; light intensity of 8 - 12 lx) for 5 min for assessment of generalized fear. The open field test was followed by a 1.5 hr rest period in the colony room and then a 5 min exposure to the shock chamber (light intensity of 400 - 500 lx) for assessment of contextual fear. The amount of time that rats spent immobility, the locomotor activity (number of floor lines crossed by the rat), and the latency it took for rats to go to the center area of the open field were calculated for the open field test whereas immobility percentage was calculated for the shock chamber exposure.

**Table 6  Timeline of the behavioral test in TCS-1102 experiment 1**

Day 0    Shock exposure

Day 1    Small open field and novel tone test

Day 14   TCS-1102 or vehicle injection, followed by big

open field test and shock chamber exposure

### 2.9.2. Statistical Analyses

A two-way ANOVA was used to evaluate the main and interaction effects of "shock" (shock or nonshock) and "TCS-1102" (vehicle, 5 mg, 10 mg or 20 mg) in behaviors expressed in the open field and the shock chamber.  When a significance was found, Student's t-test was used to compare "shock" effect on rats received the same dose of TCS-1102 injection, and a one-way ANOVA was used to analyze the effect of "TCS-1102" in the groups of shock and nonshock rats.  Fisher's LSD post-hoc tests were used to determine if differences between groups were significant in the ANOVA tests.  A value of $p < 0.05$ was considered to be significant and the data were presented as mean ± SEM.  Data analyses were performed using SPSS software, version 19.0.

## 2.10.  Effects of the Orexin Receptor Antagonist TCS-1102 on Avoidance and Escape

### 2.10.1.  HR and LR Subgroups of Shock Rats

A previous experiment has shown that the subgroup of shock rats that show a high level of acute fear responses (immobility) to a novel tone also display long-term increased anxiety-like behaviors (avoidance) when tested over a four-week period (Chen, Li et al. 2012).  The elevated T-maze (ETM) is a validated behavioral test used to evaluate the

avoidance associated with anxiety as well as assessing avoidance learning and escape tendencies (Viana, Tomaz et al. 1994; Zangrossi and Graeff 1997). The present experiment used the ETM to examine inhibitory avoidance and escape responses of nonshock, LR, and HR treated with a dual orexin receptor antagonist. The timeline of the footshock exposure and the behavioral tests is shown on Table 7. As described for the previous experiment, acute fear response to a novel tone (9 kHz, 75 dB against background noise levels of 45 - 50 dB) was assessed in rats that placed in a novel chamber (made of black Plexiglas and measuring L65 cm $\times$ W40 cm $\times$ H50 cm; light intensity of 3 - 5 lx) one day after the exposure to footshocks. The percentage of immobility was used for dividing shock rats into low responder (LR) and high responder (HR).

### 2.10.2. Drug Injection

Dual orexin receptor antagonist TSC-1102 (Tocris, Minneapolis, USA) was dissolved in Poly-ethylene glycol 200 on the day of the ETM test (PEG200, Sigma Aldrich, Canada) (Bergman, Roecker et al. 2008). A dose of 0, 5, or 10 mg/kg in a volume of 2.0 ml/kg was given to rats 30 min before the ETM test. The drug doses were chosen on the basis of the results of from previous experiments, which showed that this dose range attenuated fear and anxiety without producing sedative effects.

**Table 7  Timeline of the behavioral test in TCS-1102 experiment 2**

|  |  |
|---|---|
| Day 0 | Shock exposure |
| Day 1 | Small open field and novel tone test |
| Day 14 | TCS-1102 or vehicle injection, followed by elevated T-maze test |

### 2.10.3. Elevated T-Maze (ETM)

The ETM was made of black Plexiglass and consisted of two open arms (L 50 cm $\times$ W 10 cm $\times$ H 0.5 cm) and one closed arm (L50 cm $\times$ W10 cm $\times$ H30 cm). The maze was elevated 50 cm above the floor and placed in a dimly lit room (open arm illuminated at 5 lx and closed arm illuminated at 3 lx). The ETM test was conducted as described in previous studies (Graeff, Viana et al. 1993; Zangrossi and Graeff 1997). Rats were transferred to the test room one at a time and allowed 2 min to adapt, and then they were placed in the end of closed arm of the ETM facing the intersection of the arms and the time taken to enter the open arm with the four paws was recorded as the baseline latency. The trial was terminated after 5 min and repeated in two subsequent trials (avoidance 1 and 2) with a rest interval in a holding cage for 30 s between each trial. After that, the rat was placed in the end of one open arm in two consecutive trial for recording the time taken to withdraw from the open arm with the four paws crossed into the closed arm (escape 1and 2). As before, the rest interval between the escape trials was 30 s.

### 2.10.4. Statistical Analyses

Student's t-tests were used for analysis of immobility between shock and nonshock rats (grouping data). All the ETM results were subjected to a logarithmic transform for avoiding the heterogeneity. Comparisons between the trials in the ETM were analyzed by a three-factor ANOVA design, with the "shock" groups (nonshock, LR,

HR) and "TCS-1102" doses (0, 5, and 10 mg/kg) as two independent factors and "trial" (baseline, avoidance 1 and 2; or escape 1 and 2) as the dependent factor. Whenever a significant effect was found for "shock" or interaction between "shock" and other factors, the one-way ANOVA were made to compare the groups of nonshock, LR, and HR received similar treatment at each trial session and Fisher's LSD post-hoc test to compare the specific group difference if necessary. In case of significant effects of "TCS-1102" or interaction between "TCS-1102" and other factors, the one-way ANOVA were performed to investigate the dose effect of the drug on latency within nonshock or shock groups at each trial. If a significant effect was found on "trial" or interaction between "trial" and other factors, the repeated one-way ANOVA was used to analyze the latency within shock or nonshock group treated with similar dose of TCS-1102. A value of $p < 0.05$ was considered to be significant and the data were presented as mean $\pm$ SEM. Data analyses were performed using SPSS software, version 19.0.

# Chapter 3

# Results

## 3.1. A Rat Model of PTSD

### 3.1.1. Acute Fear Response to Novel Environment

These experiments were done to test the hypothesis that the acute generalized fear response in rats that were exposed a brief episode of moderately intense footshocks predicts the long-term expression of anxiety. One day after the shock exposure, nonshock and shock rats were placed in a small open field for a period of time with the absence (3 min) and presence (3 min) of a novel tone. The Student's t-test revealed that shock rats showed more immobility during the initial exposure to the small open field ($t_{44}$ = 7.82, p < 0.001; Figure 2a) and when the novel tone was presented ($t_{44}$ = 7.41, p < 0.001; Figure 2a). The total amount of immobility expressed when shock rats were placed in the small open field ranged from 0 to 74% of the time, whereas the response to the tone ranged from 0 to 100% (Figure 2b). We used the immobility response to the novel tone to subdivide shock rats into LR (immobility < 40%) and HR (immobility > 60%) groups (Figure 2c), and the remainder of shock rats (immobility percentage ranging 40 - 60%) were omitted from further analysis. HR displayed more immobility when they were compared to LR ($t_{16}$ = 4.92, p < 0.001; Figure 2c).

**Figure 2  Acute fear response to novel environment**


Acute fear response (immobility) to a novel chamber and a novel tone one day after the shock exposure.  (a) Immobility response to the novel small open field and the novel tone in nonshock (n = 22) and shock rats (n = 24).  Shock rats showed an increase in immobility responses.  (b) Shock rats displayed a wide range of immobility responses as shown in plots of individual immobility responses to the small open field before and after the presence of the novel tone.  (c) Shock rats were subdivided into low responders (LR, immobility < 40%, n = 8) and high responders (HR, immobility > 60%, n = 10) based on their immobility responses to the novel tone.  The values in the histograms are mean ± SEM. *** $p < 0.001$.
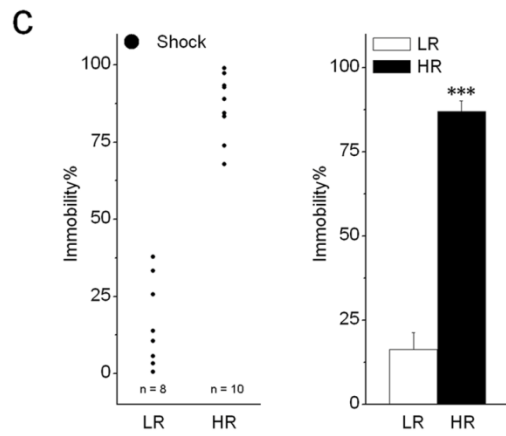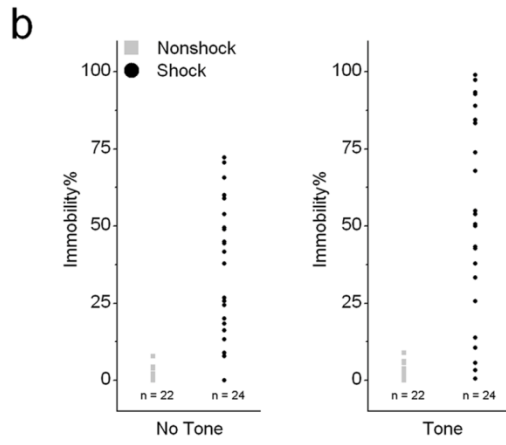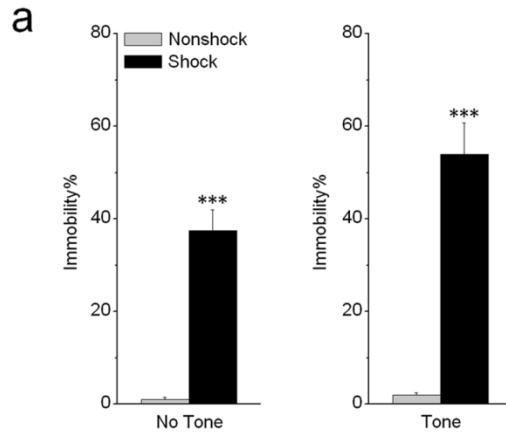
### 3.1.2. Fear Response to Novel Chambers and the Shock Chamber

Generalized fear was assessed by measuring the amount of immobility displayed when rats were placed in a novel chamber or open field on Days 11 and 26, respectively. The Kruskal-Wallis test indicated group differences on Day 11 (H = 24.00, $p < 0.001$; Figure 3a) and the comparison between different groups indicated that HR expressed more immobility than nonshock rats ($p < 0.001$) and LR ($p < 0.05$) and that LR expressed more immobility more than nonshock rats ($p < 0.01$). The number of USV were recorded when rats were exposed to the novel context on Day 11 and the Kruskal-Wallis test revealed significant differences on emission of USV (H = 17.28, $p < 0.001$; Figure 3a) between nonshock, LR and HR groups. The nonparametric t-test showed HR emitted more USV in the range of 20 - 30 kHz than nonshock rats ($p < 0.001$) and LR ($p < 0.05$) and LR showed more USV compared to nonshock rats ($p < 0.05$). The Kruskal-Wallis analysis also revealed group difference on the immobility expression on Day 26 (H = 6.87, $p < 0.05$; Figure 3a) with HR showing more immobility than nonshock rats ($p < 0.05$) and LR ($p < 0.01$). However, no difference was detected between nonshock rats and LR ($p > 0.05$) on immobility expressed on Day 26.

Fear expression when rats are re-exposed to the shock chamber was used to assess the immobility response to the context. The Kruskal-Wallis test revealed group differences of immobility expression on Day 12 (H = 29.20, $p < 0.001$; Figure 3b). The nonparametric t-test showed HR spent more time immobility than nonshock rats ($p < 0.001$) and LR ($p < 0.05$) and LR showed more immobility compared to nonshock rats ($p < 0.001$). Group differences in the number of USV emitted were also detected

**Figure 3  Fear responses to novel chambers and the shock chamber**

Immobility and ultrasonic vocalization (USV) emissions in nonshock rats, low responders (LR) and high responders (HR) exposed to novel chambers and the shock chamber at mid (Days 11 & 12) and late time points (Days 26 & 27).  (a) Both HR and LR showed increased immobility and USV emissions to the novel chamber compared to nonshock rats at Day 11.  HR still showed increased immobility at Day 26, but LR showed similar level of immobility to nonshock rats.  (b) HR and LR showed increased immobility to the shock chamber compared to nonshock rats, and only HR emitted more USV at both test Days 12 & 27.  Values are mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to nonshock group; # $p < 0.01$, ## $p < 0.001$ compared to LR group.

Novel Chamber

Shock Chamber
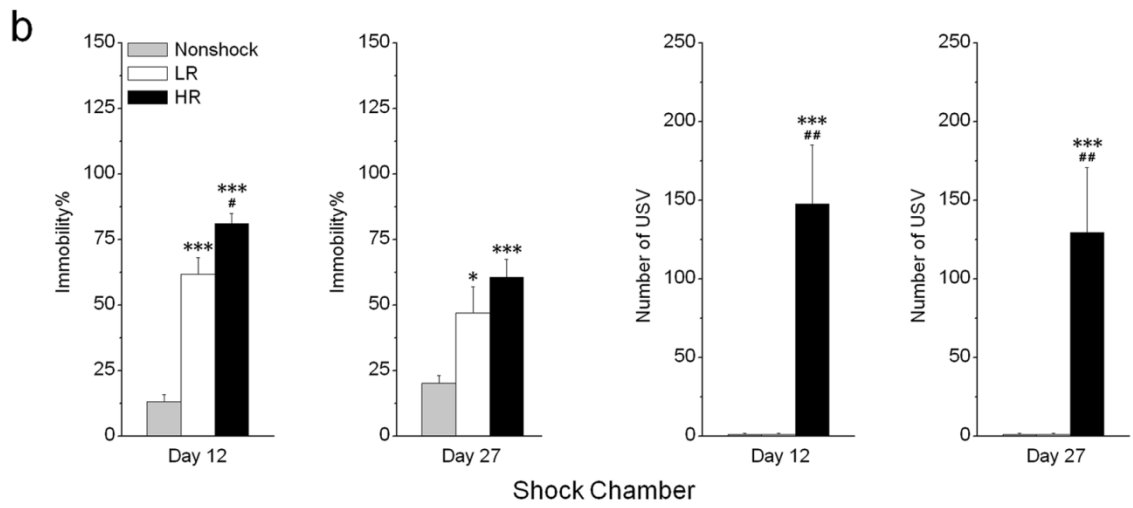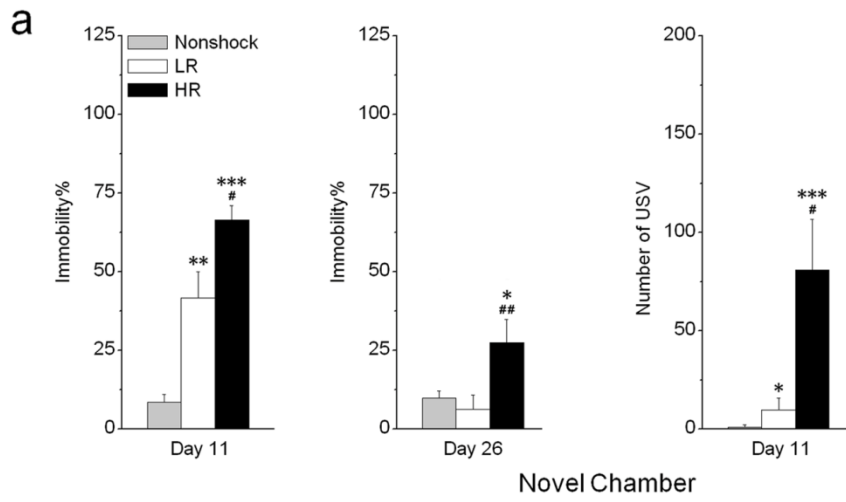
by Kruskal-Wallis analysis (H = 24.63, p < 0.001; Figure 3b) with HR showing more USV than nonshock rats (p < 0.001) and LR (p < 0.01).  The Kruskal-Wallis test also demonstrated immobility differences between the groups on the test Day 27 (H = 15.31, p < 0.001; Figure 3b) with both HR (p < 0.001) and LR (p < 0.05) showing more immobility when they were compared to nonshock rats.  The Kruskal-Wallis analysis also indicated group differences in the number of USV emissions (H = 16.79, p < 0.001; Figure 3b) with HR emitted more USV than nonshock (p < 0.001) and LR (p < 0.01) rats.

### 3.1.3.  Avoidance Behaviors

Avoidance of a novel rat was measured using a social interaction paradigm on Days 10 and 25.  The Kruskal-Wallis test revealed significant difference between groups in the latency of entering the interaction zone on the test Day 10 (H = 11.83, p < 0.01; Figure 4a).  Further analysis indicated that HR took a longer time compared to nonshock rats (p < 0.001) and LR (p < 0.008) to enter the chamber adjacent to the novel rat. Similar group differences were obtained on Day 25 (H = 11.58, p < 0.01; Figure 4a) with HR showing a longer latency than nonshock rats (p < 0.01) and LR (p < 0.01). Avoidance of open spaces from a secure area was examined using the defensive withdrawal test on Day 31.  The Kruskal-Wallis test also indicated group differences in the latency to exit the secure small chamber and enter the large open field (H = 15.24, p < 0.001; Figure 4b).  Subsequent analysis showed that HR took longer than nonshock rats (p < 0.001) and LR (p < 0.01) to enter the large open field.

**Figure 4  Avoidance behaviors**

Avoidance to a novel rat (social interaction test) or open spaces (defensive withdrawal test) in nonshock, low responders (LR) and high responders (HR).  (a) Only HR showed a strong avoidance tendency in the social interaction test at test Days 10 & 25, whereas LR displayed an avoidance latency that was similar to nonshock rats.  (b) Only HR showed a strong avoidance tendency in the defensive withdrawal test at test Days 31.  Values are mean $\pm$ SEM. ** $p < 0.01$, *** $p < 0.001$ compared to nonshock group; ## $p < 0.01$ compared to LR group.
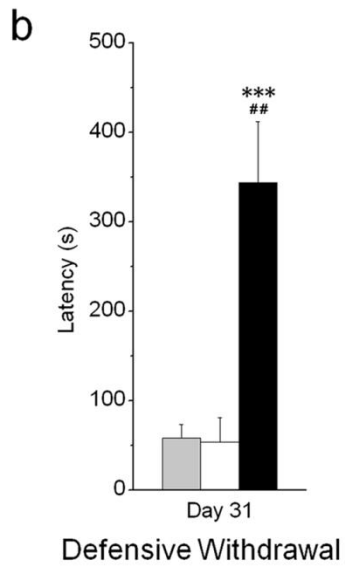
a

Social Interaction

b

Defensive Withdrawal
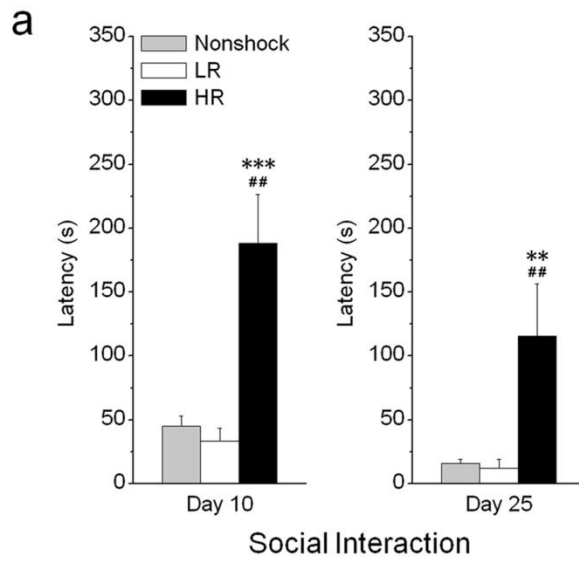
In conclusion, the results of the present experiment demonstrate that a moderated intense footshock produce rats that showed an increase in fear and anxiety for a four-week period. Shock rats that exhibited a high level of fear to the novel tone (HR) one day after the shock exposure showed more avoidance-like behavior compared to shock rats that exhibited a low level of fear to the novel tone (LR). These results highlight the importance of individual difference in long-term expression of anxiety and provide further validation of the rodent footshock as a useful model to study the mechanisms of PTSD.

## 3.2. Fear Extinction Experiments

### 3.2.1. Fear Extinction Training

The present experiments were done to examine whether fear extinction in rats that were pre-exposed to footshocks interferes with the fear expression to a novel chamber and a novel tone. Rats were placed in the shock chamber one day after the shock exposure to examine their immobility response to shock context. Based on this immobility, the shock rats were assigned to shock/control and shock/extinction groups in a way that the subgroup would have displayed similar immobility in the shock chamber. As expected, statistically analysis of these group using a one-way ANOVA demonstrated that there was an effect of shock on immobility ($F_{(2,57)} = 175.61$, $p < 0.001$; Figure 5a),

**Figure 5  Effect of fear extinction on fear response to novel environment**

Effect of fear extinction on fear response to a novel chamber and a novel tone in nonshock (n = 20), shock/control (n = 20), shock/extinction (n = 20) rats.  (a) Immobility response to the shock chamber at Days 1 and 13 before and after extinction training (Day 7 - 10).  (b) Immobility response to the shock chamber during extinction training. Shock/extinction rats show similar levels of immobility with nonshock rats at the end.  (c) Fear extinction decreased immobility to the novel chamber and during the presentation of the novel tone at Day 15.  Shock/extinction group also showed an increase in the locomotor activity displayed to the novel chamber and the novel tone compared to shock/control group.  Values are mean $\pm$ SEM.  * indicates $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to nonshock group; ## $p < 0.01$, ### $p < 0.001$ compared to shock/control group.
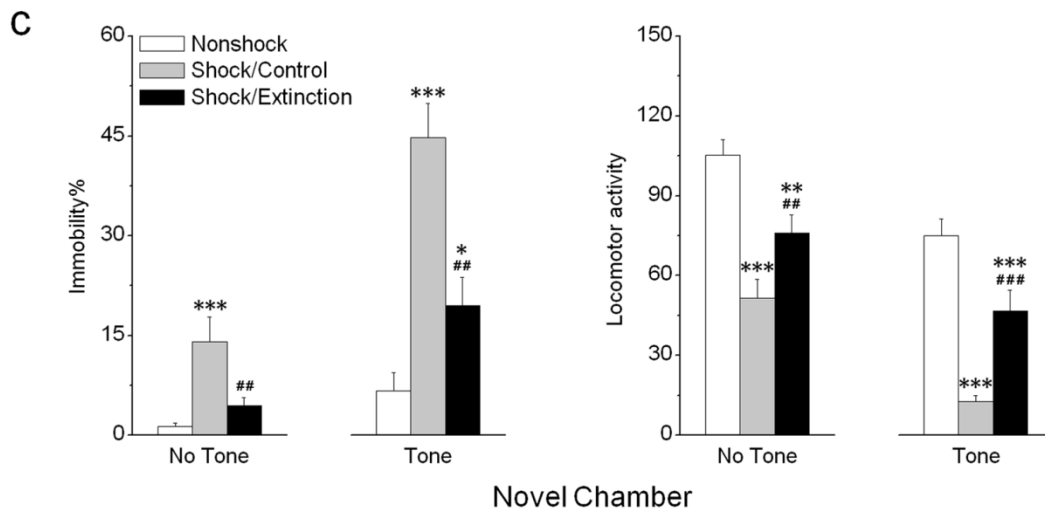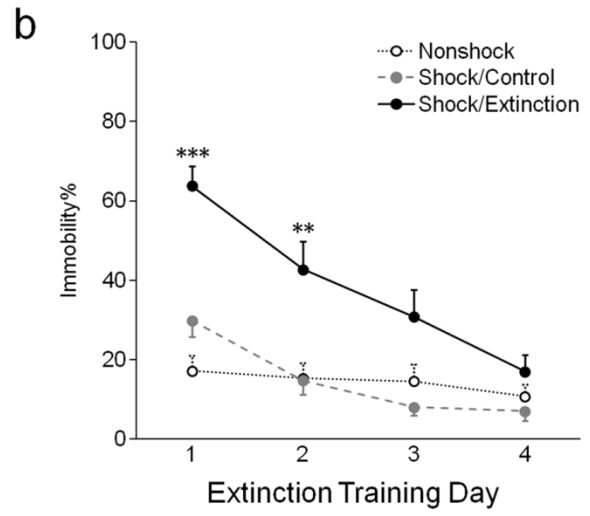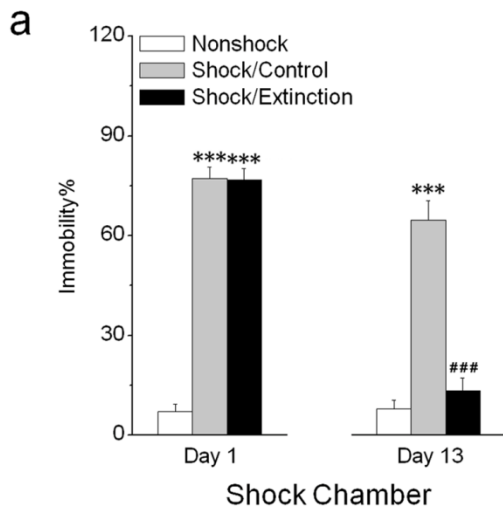
and the post-hoc analysis showed shock/control ($p < 0.001$) and shock/extinction ($p < 0.001$) groups showed increased immobility compared to nonshock rats.

During the extinction exposure, shock/extinction group showed increased immobility to the shock chamber at Day 1 ($t_{38} = 7.36$, $p < 0.001$; Figure 5b) and Day 2 ($t_{38} = 3.42$, $p < 0.01$; Figure 5b), but exhibited similar amount of immobility with nonshock rats at Day 3 ($t_{38} = 2.01$, $p > 0.05$; Figure 5b) and Day 4 ($t_{38} = 1.26$, $p > 0.05$; Figure 5b). Rats were returned to the shock chamber on Day 13 to examine if the extinction of the fear was still present. A significant effect of shock was found in that analysis ($F_{(2,57)} = 52.48$, $p < 0.001$; Figure 5a) and the post-hoc analysis revealed that shock/control rats still displayed a higher level of immobility compared to nonshock ($p < 0.001$) and shock/extinction rats ($p < 0.001$). In addition, shock/extinction group showed similar amount of immobility with nonshock rats ($p > 0.05$).

### 3.2.2. Effects of Extinction on Fear Response to Novel Environment

After the fear extinction training, the rats were exposed to a novel chamber and a novel tone at Day 15. The one-way ANOVA showed a significant effect of shock on immobility ($F_{(2,57)} = 8.30$, $p < 0.001$; Figure 5c) when they were placed in a novel chamber and the post-hoc analysis revealed that the shock/control rats showed increased immobility to the novel chamber compared to nonshock rats ($p < 0.001$) and to the shock/extinction rats ($p < 0.01$). There was no difference between the immobility produced by nonshock rats and shock/extinction rats ($p > 0.05$). When a novel tone was

present, the one-way ANOVA revealed a significant effect of shock on immobility ($F_{(2,57)}$ = 21.73, p < 0.001; Figure 5c). The post-hoc analysis suggested that the shock/control rats displayed increased immobility compared to nonshock (p < 0.001) and shock/extinction groups (p < 0.01). Furthermore, the shock/extinction rats showed elevated immobility compared to nonshock rats (p < 0.05).

The one-way ANOVA also showed an effect of shock on locomotor activity ($F_{(2,57)}$ = 17.12, p < 0.001; Figure 5c) when rats were exposed to the novel chamber. The post-hoc analysis revealed that shock/control (p < 0.001) and shock/extinction (p < 0.01) rats showed a decrease in locomotor activity compared to nonshock rats. The extinction training increased the locomotor activity (p < 0.01) in shock/extinction rats compared to shock/control rats. Under conditions when the novel tone was presented, the one-way ANOVA revealed an effect of shock on locomotor activity ($F_{(2,57)}$ = 27.53, p < 0.001; Figure 5c). Shock/control (p < 0.001) and shock/extinction groups (p < 0.001) showed a decrease in locomotor activity compared to nonshock ones. Shock/extinction rats also displayed an increase in locomotor activity (p < 0.001) compared to shock/control rats.

In summary, fear extinction training by repeatedly placing rats in the shock chamber attenuated the fear expressed to the novel chamber. No difference was detected between the shock/extinction groups and the nonshock rats. This indicates that the expression of fear in the novel chamber is mainly mediated by fear generalization. However, based on the fact that the shock/extinction group had an elevated immobility response to the novel tone when compared to nonshock rats, which suggests that some fear sensitization is involved in shock/extinction group.

### 3.3. Effects of Footshock on ppOX mRNA Levels

### 3.3.1. Levels of ppOX mRNA in Rats at 6 Days Post-shock

In order to examine the short term effect of the shock exposure on ppOX mRNA levels, rats were exposed to a single episode of footshocks, and their fear response to a novel chamber and the shock chamber were recorded during 1-2 days after the initial shock procedure. Rats were then decapitated and their brains were removed for assessment of ppOX mRNA using in situ hybridization. Rats exposed to footshocks showed more immobility compared to nonshock rats when they were placed in the novel chamber ($t_{13} = 6.09$, $p < 0.001$; Figure 6a) and in the shock chamber ($t_{13} = 6.60$, $p < 0.001$; Figure 6a). The levels of ppOX mRNA was increased in shock rats ($t_{13} = 3.92$, $p < 0.01$; Figure 6b) compared to nonshock rats. Specifically, ppOX mRNA levels were elevated in the medial portion ($t_{13} = 3.54$, $p < 0.01$; Figure 6b) and the lateral portion of the orexin population ($t_{13} = 2.40$, $p < 0.05$; Figure 6b) in shock rats. In addition, the Pearson correlation analysis indicated that levels of ppOX mRNA were positively correlated with the immobility expressed to the novel chamber ($R^2 = 0.50$, $p < 0.01$) or the shock chamber ($R^2 = 0.50$, $p < 0.01$) when the data of both the shock and nonshock rats were plotted together. However, there is no relationship between ppOX mRNA levels and immobility response to the novel chamber (shock: $R^2 = 0.19$, $p > 0.05$; nonshock: $R^2 = 0.10$, $p > 0.05$; Figure 6c) or the shock chamber (shock: $R^2 = 0.17$, $p > 0.05$; nonshock: $R^2 = 0.003$, $p > 0.05$; Figure 6d) when the data were analyzed as shock or nonshock group.

**Figure 6  Levels of ppOX mRNA in rats at 6 days post-shock**

Fear and prepro-orexin (ppOX) mRNA levels at 6 days after the shock exposure in shock (n = 8) and nonshock rats (n = 7).  (a) Shock rats showed an increase in immobility to a novel chamber (generalized fear) at Day 1 and the shock chamber (contextual fear) at Day 2.  (b) Shock rats showed an increase in ppOX mRNA levels in the hypothalamus including the medial and lateral parts of orexin population.  (c) There was no correlation between ppOX mRNA levels and immobility expressed in nonshock or shock rats that were exposed to the novel chamber.  (d) There was no correlation between ppOX mRNA levels and immobility expressed in nonshock or shock rats that were exposed to the shock chambers.  Values are indicated as mean $\pm$ SEM for (a) and (b).  Significant difference between groups with * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.
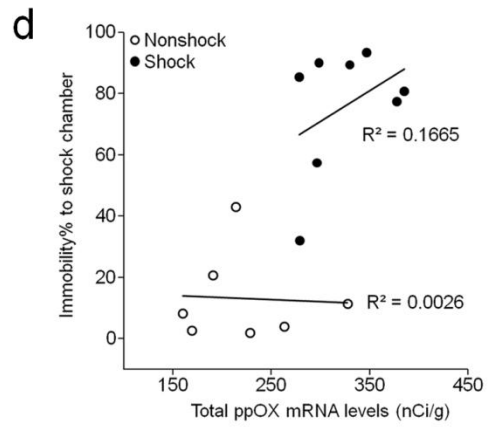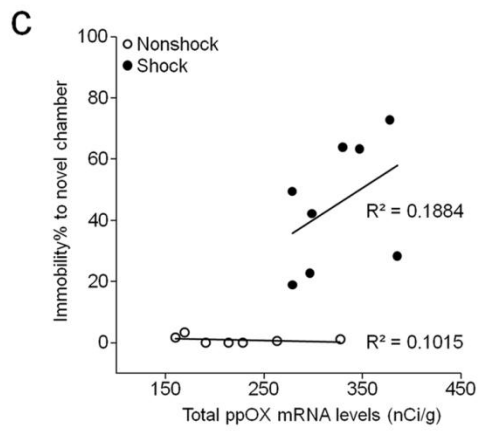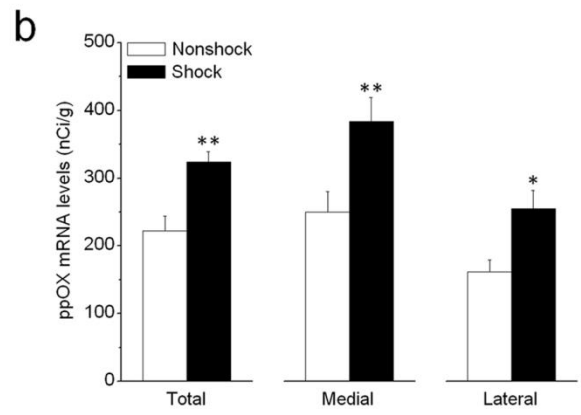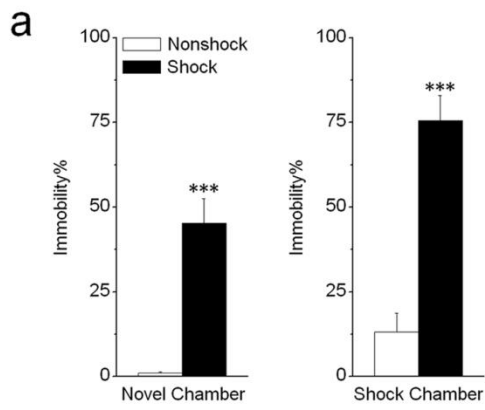
### 3.3.2. Levels of ppOX mRNA in Rats at 14 Days Post-shock

Animals used for examination of the ppOX mRNA levels at 14 days post-shock were exposed to a novel chamber on Day 9 and the shock chamber on Day 10. Shock rats expressed more immobility to the novel chamber ($t_{15}$ = 3.60, p < 0.01; Figure 7a) and the shock chamber ($t_{15}$ = 4.66, p < 0.001; Figure 7a) compared to nonshock rats. Shock rats also expressed higher levels of ppOX mRNA ($t_{15}$ = 3.07, p < 0.01; Figure 7b) compared to nonshock rats. Both of the medial part ($t_{15}$ = 2.91, p < 0.05; Figure 7b) and lateral part ($t_{15}$ = 2.86, p < 0.05; Figure 7b) of orexin population in shock group showed increased ppOX mRNA levels. The levels of ppOX mRNA were positively correlated with immobility response to the novel chamber ($R^2$ = 0.56, p < 0.001) and the shock chamber ($R^2$ = 0.67, p < 0.001) when the data of both the shock and nonshock rats were plotted together. In addition, a significant correlation was found between the ppOX mRNA levels and immobility in shock rats when they were exposed to the shock chamber ($R^2$ = 0.50, p < 0.05; Figure 7d) which was not present in nonshock rats ($R^2$ = 0.01, p > 0.05; Figure 7d). No correlation was found in ppOX mRNA levels and immobility expressed to the novel chamber (shock group: $R^2$ = 0.34, p > 0.05; nonshock group: $R^2$ = 0.05, p > 0.05; Figure 7c).

In summary, rats that were previously exposed to moderated intense footshocks showed more immobility compared to nonshock rats when they were placed in a novel chamber (generalized fear) and the shock chamber (contextual fear). The levels of ppOX mRNA in the medial and lateral portion of orexin population were increased in shock rats at both 3 and 14 days post-shock. Interestingly, the levels of ppOX mRNA expressed

**Figure 7  Levels of ppOX mRNA in rats at 14 days post-shock**

Fear and prepro-orexin (ppOX) mRNA levels at 14 days after the shock exposure in shock (n = 9) and nonshock rats (n = 8).  (a) Shock rats showed an increase in immobility to a novel chamber (generalized fear) at Day 9 and the shock chamber (contextual fear) at Day 10.  (b) Shock rats showed an increase in ppOX mRNA levels in the hypothalamus which included the medial and lateral parts of orexin population.  (c) There was no correlation between ppOX mRNA levels and immobility expressed in shock or nonshock rats that were placed in the novel chamber.  (d) There was a positive correlation between ppOX mRNA levels and immobility expressed to the shock chamber in shock rats.  Values are indicated as mean $\pm$ SEM for (a) and (b).  Significant difference between groups with * $p < 0.05$; ** $p < 0.01$.
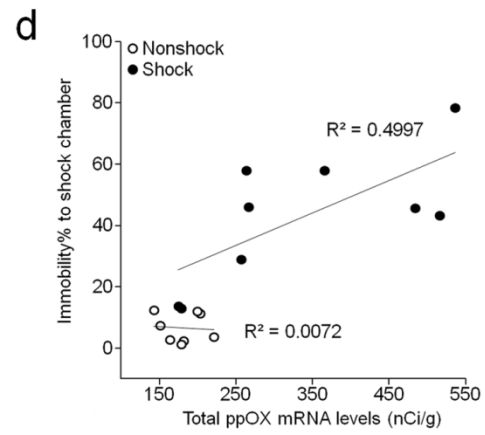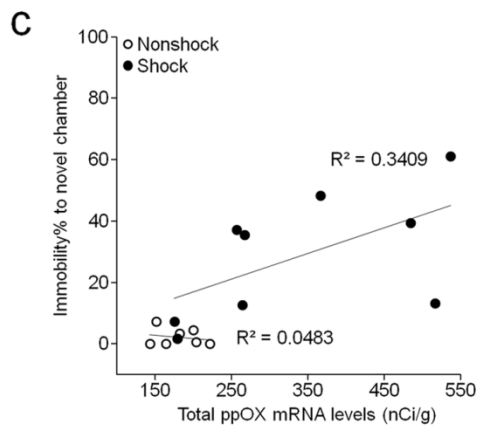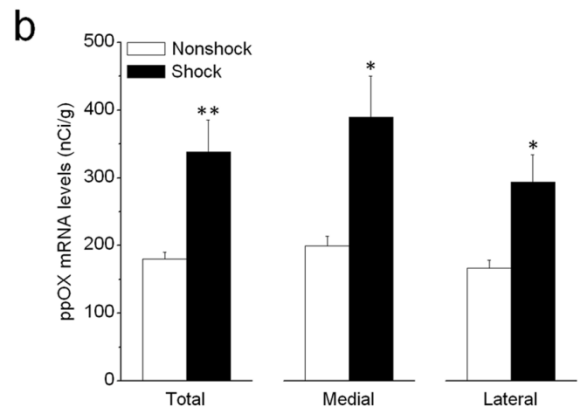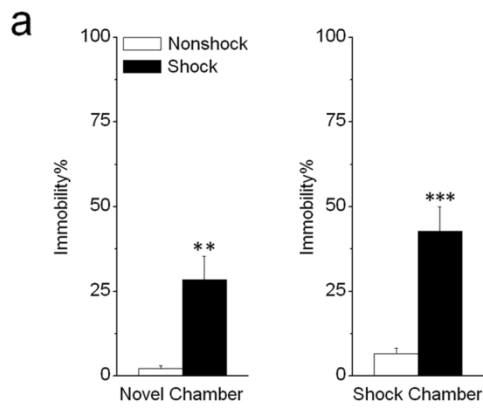
at Day 14 after the shock exposure were found to be correlated with the contextual fear expressed in the shock animals.

## 3.4. Changes in the Activity of Orexin Neurons During Expression of Fear

### 3.4.1. Fear Response to a Novel Chamber or the Shock Context

These experiments were done to determine whether orexin neurons are activated when rats that were pre-exposed to footshocks are placed in a new home cage, a novel chamber or the shock chamber. First of all, there was no group differences in the time spent in the shading ($t_{13} = 0.48$, $p > 0.05$; Figure 8a) or the durations of rearing ($t_{13} = 1.21$, $p > 0.05$; Figure 8a) between shock and nonshock groups when they were remained in their home cage. However, shock rats showed higher immobility ($t_{16} = 4.68$, $p < 0.001$; Figure 8b) and decreased duration of rearing ($t_{16} = 3.19$, $p < 0.01$; Figure 8b) compared to nonshock rats when they were exposed to the novel chamber. Similarly, shock rats displayed increased immobility ($t_{20} = 9.25$, $p < 0.001$; Figure 8c) and decreased durations of rearing ($t_{20} = 2.96$, $p < 0.01$; Figure 8c) when they were exposed to the shock chamber. This indicates that shock rats displayed fear when they were placed in a novel and shock chamber but not in the home cage.

**Figure 8  Behavioral responses to the exposure chamber**


Fear response in rats exposed to a new home cage (shock, n = 8; nonshock, n = 7), a novel chamber (shock, n = 9; nonshock, n = 9) or the shock chamber (shock, n = 11; nonshock, n = 11) 14 days after the shock exposure.  (a) Duration of hiding in the shading or rearing when rats were exposed to the home cage.  Percentage of immobility and rearing when rats were exposed to the novel chamber (b) or the shock chambers (c). Shock rats showed similar levels of hiding and rearing when they were placed in the home cage, and increased immobility to the novel and shock chamber.  Values are mean $\pm$ SEM.  Significant difference between groups with ** $p < 0.01$, *** $p < 0.001$.
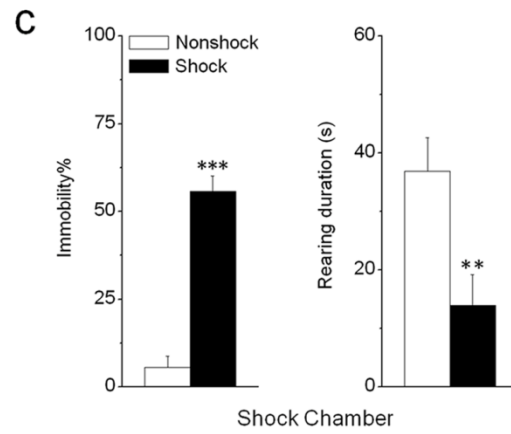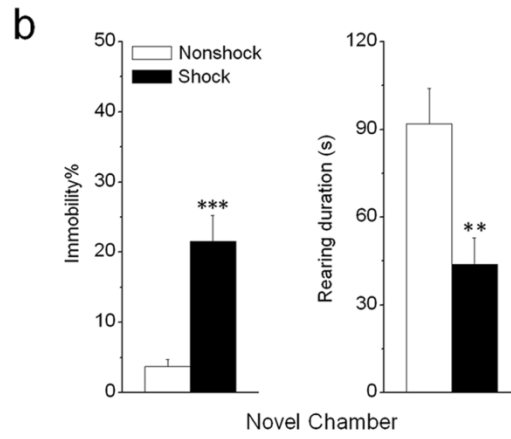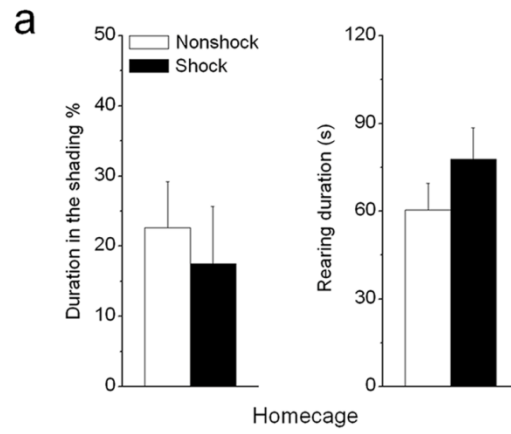
### 3.4.2. Changes in the Activity of Orexin Neurons During Expression of Fear

Rats were anesthetized and perfused 14 days after the footshock exposure and the exposure to either home cage, novel chamber, or shock chamber. The brains were collected for immunohistochemical staining and quantification of cFos-positive orexin neurons (as shown in Figure 9). There were no differences in the total number of orexin neurons counted for the different groups and conditions examined: numbers for nonshock rats placed in the home cage (698 $\pm$ 26); novel chamber (736 $\pm$ 24); and shock chamber (695 $\pm$ 14) were similar to shock rats placed in the home cage (666 $\pm$ 23); novel chamber (706 $\pm$ 27); and shock chamber (694 $\pm$ 21). The two-way ANOVA indicated that there was a main effect of "exposure places" ($F_{(2,49)} = 10.89$, $p < 0.001$) on the number of cFos/orexin double-labeled neurons. However, there was no effect for "shock" ($F_{(1,49)} = 1.38$, $p > 0.05$) nor was there an interaction effect between "exposure places" and "shock" ($F_{(2,49)} = 0.52$, $p > 0.05$). The one-way ANOVA revealed a significant difference in the number of cFos/orexin double-labeled neurons in nonshock rats placed in different chambers ($F_{(2,24)} = 9.59$, $p < 0.001$; Figure 10a). The post-hoc analysis indicated an increase of orexin neuron activity in nonshock rats which were placed in either the novel chamber ($p < 0.001$) or the shock chamber ($p < 0.01$) compared to rats placed in the home cage. A similar effect was also detected in shock rats ($F_{(2,25)} = 3.39$, $p < 0.05$; Figure 10a) when they were exposed to different places, and the post-hoc analysis showed the activity of orexin neurons was increased in shock rats that were placed in the novel chamber ($p < 0.05$) and the shock chamber ($p < 0.05$).

**Figure 9  Photomicrograph of the lateral hypothalamus showing the orexin neurons and cFos protein expression**


Example of medial posterior hypothalamic coronal sections selected from shock rats exposed to the home cage (a), novel chamber (b) and shock chamber (c).  Arrows illustrate double immunohistochemical staining of cFos protein expressing nucleus (black) and orexin neurons (brown).

a

Fornix                    200 μm

Home Cage

b

Fornix                    200 μm

Novel Chamber

c

Fornix                    200 μm

Shock Chamber

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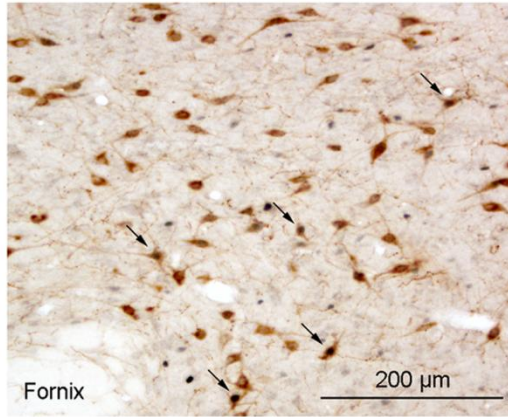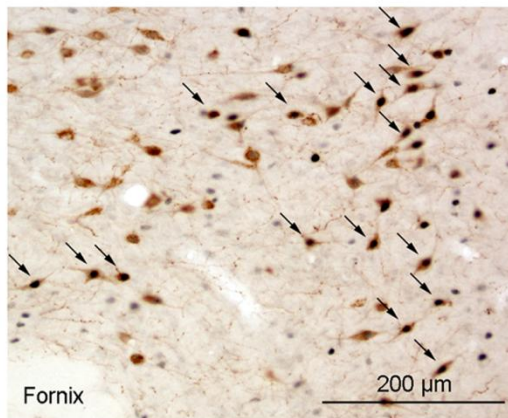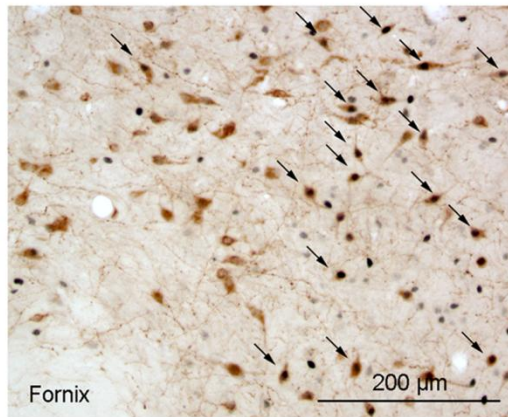
**Figure 10  Changes in the activity of orexin neurons during fear expression**

Changes in the cFos expression in orexin neurons during the chambers exposure.  (a) Percentage of cFos/orexin double-labeled neurons in rats exposed to a new home cage (shock n = 8; nonshock n = 7), a novel chamber (shock n = 9; nonshock n = 9) or the shock chamber (shock n = 11; nonshock n = 11) 14 days after the shock exposure.  The number of cFos/orexin double labeled neurons was increased when shock and nonshock rats were placed in the novel and shock chamber.  Double-labeled neurons in medial part (b) and lateral part (c) of orexin population were also calculated when rats were exposed to the different chambers.  Values are mean $\pm$ SEM.  Significant difference between groups with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.  * in Figure c presents comparison with rats exposed to the home cage.
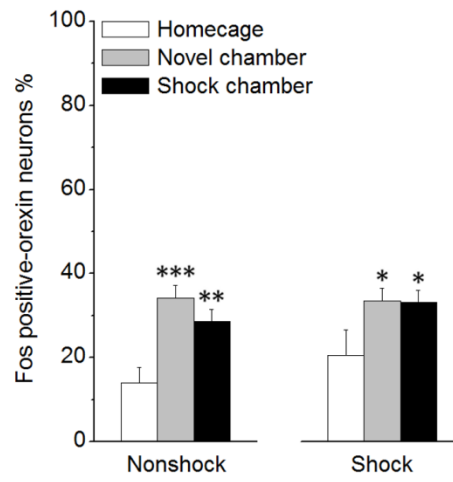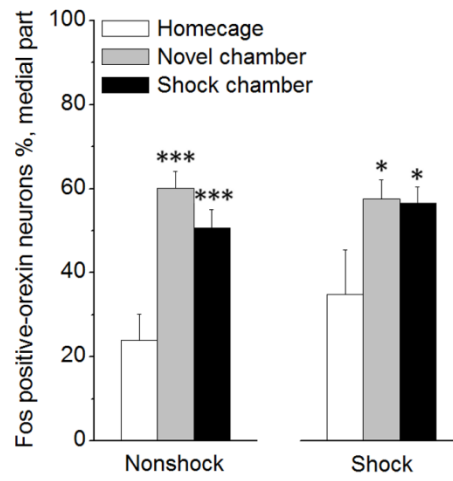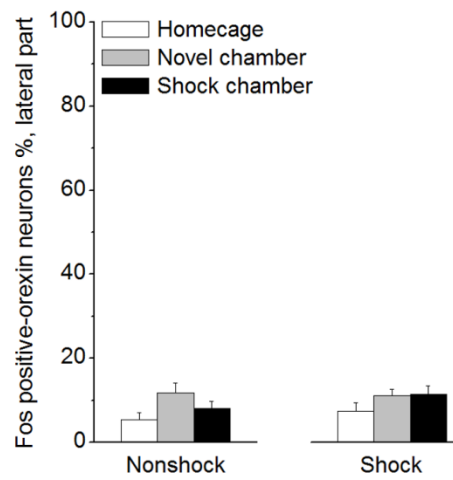
As described before, the medial and lateral populations of orexin neuron have been shown to be functionally different. In order to examine this function dichotomy, the percentage of Fos-positive orexin neuron was scored separately for the medial and lateral populations of orexin neuronal. The two-way ANOVA suggested a main effect of "exposure places" on the number of double-labeled neurons in the medial portion of the orexin population ($F_{(2,49)} = 13.72$, $p < 0.001$). However, no main effect for "shock" ($F_{(1,49)} = 1.10$, $p > 0.05$) or interaction between "shock" and "exposure places" ($F_{(2,49)} = 0.67$, $p > 0.05$) was found. The one-way ANOVA showed that the number of double-labeled neurons in the medial orexin population was increased in both nonshock ($F_{(2,24)} = 13.20$, $p < 0.001$; Figure 10b) and shock rats ($F_{(2,25)} = 3.79$, $p < 0.05$; Figure 10b) when they were placed in different chambers. The post-hoc analysis revealed that the medial population of orexin neurons had more double-labeled neurons in nonshock rats placed in either the novel chamber ($p < 0.001$) or the shock chamber ($p < 0.001$) as compared to the home cage. Similarly, shock rats also showed increased number in double-labeled neurons in the medial population when placed in the novel ($p < 0.05$) and the shock chamber ($p < 0.05$). At last, no statistical differences in the number of double-labeled neurons in the lateral population of orexin neurons was found when shock and nonshock rats were placed in different conditions ("shock": $F_{(1,49)} = 0.92$, $p > 0.05$; "exposure places": $F_{(2,49)} = 3.15$, $p > 0.05$; interaction effect: $F_{(2,49)} = 0.62$, $p > 0.05$; Figure 10c).

To sum up, more orexin neurons in the medial population expressed cFos protein when nonshock rats were exposed to a novel chamber or the shock chamber. This is consistent with other observations suggesting that orexin neurons are involved in novelty arousal (Espana, Valentino et al. 2003; Furlong, Vianna et al. 2009) and that medial

population of orexin neurons is more likely to be associated with arousal and stress (Harris, Wimmer et al. 2005; Harris and Aston-Jones 2006).

## 3.5. Effects of the CRF Receptor-1 Antagonist Antalarmin on Fear and ppOX mRNA Levels

### 3.5.1. Effects of Antalarmin on the Immediate Fear Response to Footshocks

Previous studies have demonstrated that stress-induced release of CRF activates orexin neurons. The present experiment examined the effect of a CRF receptor-1 antagonist on fear expression and ppOX mRNA levels two weeks after the initial stress episode. The immobility percentage and number of USV emitted when rats were exposed to footshocks were recorded. The two-way ANOVA revealed a significant effect of "shock" on immobility ($F_{(1,35)} = 518.75$, $p < 0.001$) and number of USV calls ($F_{(1,35)} = 107.17$, $p < 0.001$). No effect of "antalarmin" or interactions between "shock" and "antalarmin" were detected on the amount of immobility or number of USV emitted during the footshock exposure. The student's t-test showed that shock rats treated with vehicle ($t_{17} = 19.46$, $p < 0.001$; Figure 11) or antalarmin ($t_{18} = 13.62$, $p < 0.001$; Figure 11) had increased immobility compared to the nonshock rats with the same treatment. Likewise, shock rats treated with the vehicle ($t_{17} = 7.47$, $p < 0.001$; Figure 11) or antalarmin ($t_{18} = 7.15$, $p < 0.001$; Figure 11) emitted more number of USV compared to nonshock rats.

**Figure 11  Effect of the CRF receptor-1 antagonist antalarmin on shock responses**

Effects of the CRF receptor-1 antagonist antalarmin on shock responses in shock (Vehicle, n = 9; Antalarmin, n = 10) and nonshock rats (Vehicle, n = 8; Antalarmin, n = 10).  Antalarmin had no effect on immobility and ultrasonic vocalization (USV) when rats were exposed to the footshocks.  Values are mean $\pm$ SEM.  *** $p < 0.001$ compared to nonshock rats with vehicle injection; ### $p < 0.001$ compared to nonshock rats with antalarmin injection.
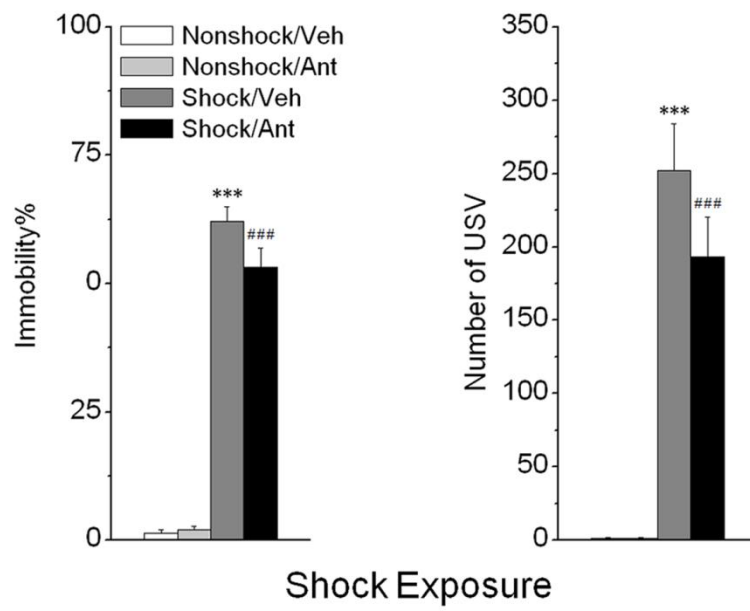
### 3.5.2. Effects of Antalarmin on Generalized Fear

The generalized fear responses to novelty was assessed by measuring the amount of immobility and locomotor activity when rats were placed in a novel chamber with presence of a novel tone on Days 1 and 9 post-shock. The two-way ANOVA indicated a "shock" effect on immobility expressed to the novel chamber ($F_{(1,35)} = 78.66$, $p < 0.001$) as well as to the novel tone ($F_{(1,35)} = 55.73$, $p < 0.001$) at Day 1. No effect of "antalarmin" or interaction between "shock" and "antalarmin" was observed. The student's t-test revealed that shock rats that had received the vehicle ($t_{17} = 6.35$, $p < 0.001$; Figure 12a) or antalarmin injection ($t_{18} = 6.17$, $p < 0.001$; Figure 12a) displayed increased immobility to the novel chamber compared to nonshock rats with the same treatment. When a novel tone is presented, vehicle ($t_{17} = 5.25$, $p < 0.001$; Figure 12a) or antalarmin-treated ($t_{18} = 5.38$, $p < 0.001$; Figure 12a) shock rats also showed increased immobility compared to nonshock rats with the same treatment.

The two-way ANOVA also revealed a significant "shock" effect on locomotor activity when rats were exposed to the novel chamber ($F_{(1,35)} = 110.27$, $p < 0.001$) or to the novel tone ($F_{(1,35)} = 153.82$, $p < 0.001$) at Day 1. No effect of "antalarmin" or interaction between "shock" and "antalarmin" was observed in immobility responses during the test. Shock rats with vehicle ($t_{17} = 6.41$, $p < 0.001$; Figure 12a) or antalarmin administration ($t_{18} = 8.41$, $p < 0.001$; Figure 12a) showed decreased locomotor activity compared to nonshock rats with similar treatment in the novel chamber. Similarly, the t-test also revealed that vehicle ($t_{17} = 7.42$, $p < 0.001$; Figure 12a) or

**Figure 12  Effect of the CRF receptor-1 antagonist antalarmin on fear responses to novel chambers**

Effects of the CRF receptor-1 antagonist antalarmin on fear responses to novel chambers in shock (Vehicle, n = 9; Antalarmin, n = 10) and nonshock rats (Vehicle, n = 8; Antalarmin, n = 10).  (a) Antalarmin had no effect on immobility and locomotor activity expressed in rats that were placed in a novel chamber before and after the presence of a novel tone at Days 1.  (b) Antalarmin increased the locomotor activity in nonshock when they were exposes to the novel chamber at Day 9. Values are mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to nonshock rats with vehicle injection; ## $p < 0.01$, ### $p < 0.001$ compared to nonshock rats with antalarmin injection.

a



Day 1

b



Day 9

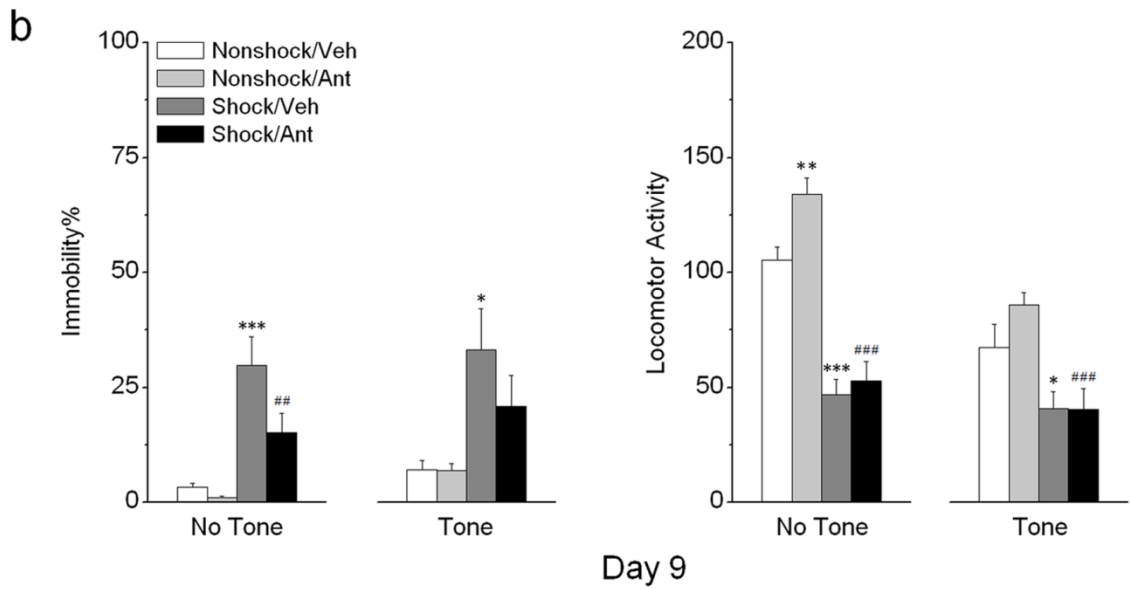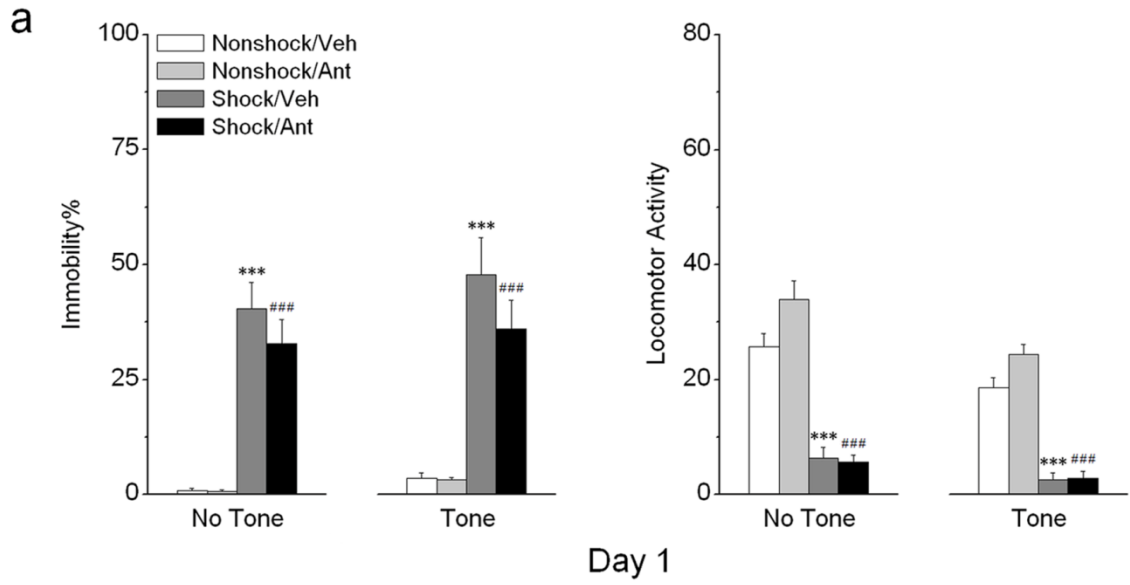antalarmin-treated ($t_{18}$ = 10.15, p < 0.001; Figure 12a) shock rats displayed decreased locomotor activity during the period when the novel tone was presented.

An effect of "shock" was found for immobility when rats were exposed to a novel chamber ($F_{(1,35)}$ = 24.72, p < 0.001) and to the novel tone ($F_{(1.35)}$ = 11.61, p < 0.01) at Day 9. There was no effect for "antalarmin" nor was there an interaction effect between "shock" and "antalarmin" on immobility responses. The student's t-test showed that vehicle ($t_{17}$ = 3.92, p < 0.001; Figure 12b) or antalarmin-treated ($t_{18}$ = 3.37, p < 0.01; Figure 12b) shock rats displayed increased immobility to the novel chamber compared to nonshock rats with the same treatment. The shock rats with vehicle injection also showed an increase in immobility to the novel tone compared to vehicle-treated nonshock rats ($t_{17}$ = 2.74, p < 0.05; Figure 12b). However, there was no difference in immobility between shock and nonshock rats that were pre-treated with antalarmin when the novel tone was presented ($t_{18}$ = 1.99, p > 0.05; Figure 12b).

Main effects of "shock" ($F_{(1,35)}$ = 98.77, p < 0.001) and "antalarmin" ($F_{(1.35)}$ = 6.123, p < 0.05) were found in locomotor activity during the exposure to the novel chamber at Day 9. There were no interaction effect between "shock" and "antalarmin" ($F_{(1.35)}$ = 2.63, p > 0.05). The student's t-test revealed a decrease in locomotor activity in the shock rats treated with vehicle ($t_{17}$ = 6.63, p < 0.001; Figure 12b) and with antalarmin ($t_{18}$ = 7.50, p < 0.001; Figure 12b) compared to the nonshock rats with similar treatment. Antalarmin increased the locomotor activity in nonshock rats ($t_{17}$ = 3.10, p < 0.01; Figure 12b), but not in shock rats ($t_{18}$ = 0.57, p > 0.05; Figure 12b). A "shock" effect ($F_{(1.35)}$ = 19.47, p < 0.001) was found in locomotor activity expressed in rats when a novel tone was presented. No effect was observed for an effect of "antalarmin" or an interaction

effect between "shock" and "antalarmin". In the vehicle ($t_{17} = 2.14$, $p < 0.05$; Figure 12b) or antalarmin-treated ($t_{18} = 4.27$, $p < 0.001$; Figure 12b) groups, shock rats showed decreased immobility to the novel tone compared to the nonshock rats with the same treatment.

### 3.5.3. Effects of Antalarmin on Contextual Fear

Rats were exposed to the shock chamber on Days 2 and 10 in order to test the effect of pre-injection of antalarmin on the contextual fear response. The two-way ANOVA demonstrated a main effect for "shock" ($F_{(1,35)} = 309.44$, $p < 0.001$), and an interaction effect between "shock" and "antalarmin" ($F_{(1,35)} = 4.19$, $p < 0.05$) on immobility to the shock chamber at Day 2. The student's t-test showed that in vehicle ($t_{17} = 16.74$, $p < 0.001$; Figure 13a) or antalarmin ($t_{18} = 9.73$, $p < 0.001$; Figure 13a) treated groups, shock rats exhibited more immobility compared to nonshock rats. The t-test also revealed that antalarmin decreased the immobility in shock rats ($t_{18} = 2.11$, $p < 0.05$; Figure 13a), but not in nonshock rats ($t_{17} = 0.14$, $p > 0.05$; Figure 13a).

Main effects for "shock" ($F_{(1,35)} = 7.47$, $p < 0.01$), "antalarmin" ($F_{(1,35)} = 4.64$, $p < 0.05$), and an interaction effect for "shock" and "antalarmin" ($F_{(1,35)} = 4.64$, $p < 0.05$) were also found for the number of USV emitted in the shock chamber at Day 2. Shock rats with vehicle injection emitted more USV compared to nonshock rats with the same treatment ($t_{17} = 2.49$, $p < 0.05$; Figure 13a). In addition, antalarmin decreased the number of USV emitted by shock rats ($t_{18} = 2.22$, $p < 0.05$; Figure 13a),

**Figure 13  Effect of the CRF receptor-1 antagonist antalarmin on fear responses to the shock chamber**

Effects of the CRF receptor-1 antagonist antalarmin on fear responses to the shock chamber in shock (Vehicle: n = 9; Antalarmin: n = 10) and nonshock rats (Vehicle: n = 8; Antalarmin: n = 10).  Antalarmin attenuated immobility and USV emitted in shock rats that were placed in the shock chamber at Days 2 (a) and Day10 (b).  Values are mean ± SEM. * $p < 0.05$, *** $p < 0.001$ compared to nonshock rats with vehicle injection; ## $p < 0.01$, ### $p < 0.001$ compared to nonshock rats with antalarmin injection; ‡ $p < 0.05$ compared to shock rats with vehicle injection.

a

Day 2

b

Day 10
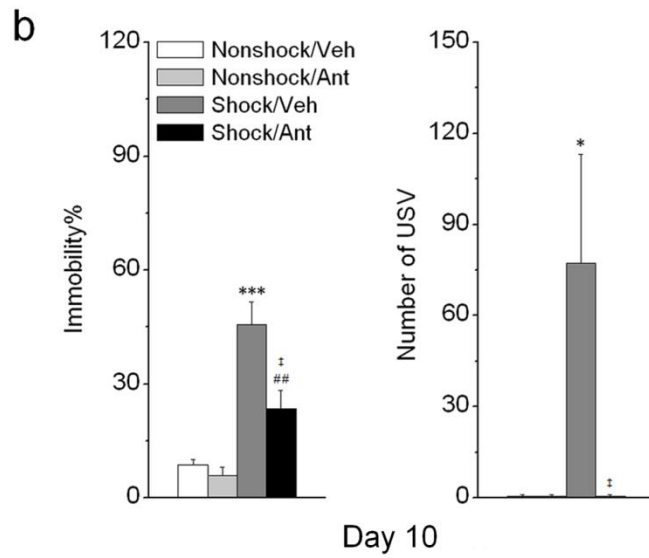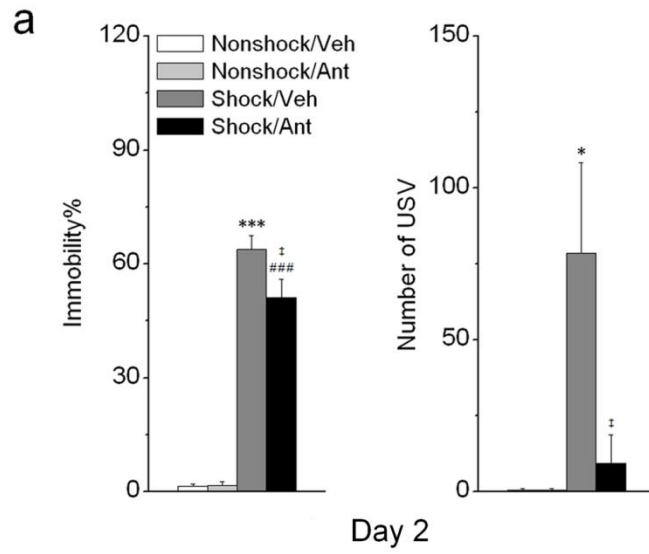
and there was no difference between the shock and nonshock rats with antalarmin treatment ($t_{18} = 1.00$, $p > 0.05$; Figure 13a).

The two-way ANOVA also revealed significant effects of "shock" ($F_{(1,34)} = 44.25$, $p < 0.001$), "antalarmin" ($F_{(1,34)} = 8.63$, $p < 0.01$) in addition to interaction effect between "shock" and "antalarmin" ($F_{(1,34)} = 5.06$, $p < 0.05$) on immobility to the shock chamber at Day 10. Shock rats with vehicle injection showed increased immobility ($t_{17} = 3.79$, $p < 0.001$; Figure 13b) compared to vehicle-treated nonshock rats. In groups with antalarmin treatment, shock rats also showed increased immobility compared to nonshock rats ($t_{17} = 3.50$, $p < 0.01$; Figure 13b). However, antalarmin decreased immobility to the shock chamber in shock rats ($t_{17} = 2.28$, $p < 0.05$; Figure 13b).

Main effects of "shock" ($F_{(1,35)} = 4.35$, $p < 0.05$), "antalarmin" ($F_{(1,35)} = 4.35$, $p < 0.05$) as well as interactions between "shock" and "antalarmin" ($F_{(1,35)} = 4.35$, $p < 0.05$) on the number of USV emitted to the shock chamber were also found for rats tested at Day 10. Vehicle-treated shock rats emitted more USV compared to vehicle-treated nonshock rats ($t_{17} = 2.16$, $p < 0.05$; Figure 13b). Antalarmin decreased the number of the USV emitted by shock rats ($t_{18} = 2.14$, $p < 0.05$; Figure 13b).

### 3.5.4. Effects of Antalarmin on ppOX mRNA Levels

Rats were perfused 14 days after the shock exposure and their brains were removed for quantification of ppOX mRNA levels using in situ hybridization. The two-way ANOVA showed an effect of "shock" on the expression of ppOX mRNA levels

($F_{(1,33)} = 19.22$, $p < 0.001$). No effect of "antalarmin" ($F_{(1,33)} = 0.09$, $p > 0.05$) or an interaction between "shock" and "antalarmin" ($F_{(1,33)} = 2.12$, $p > 0.05$) was found in the levels of ppOX mRNA. Vehicle-treated shock rats had increased ppOX mRNA levels compared to vehicle-treated nonshock rats ($t_{15} = 3.50$, $p < 0.01$; Figure 14a). Similarly, antalarmin-treated shock rats had increased ppOX mRNA levels relative to its respective control ($t_{15} = 2.48$, $p < 0.05$; Figure 14a). The two-way ANOVA also showed a "shock" effect on ppOX mRNA levels in the medial portion ($F_{(1,33)} = 24.27$, $p < 0.001$) and the lateral portion ($F_{(1,33)} = 9.49$, $p < 0.01$) of the orexin population. However, the ANOVA did not reveal an effect for "antalarmin" nor did it show an interaction between "shock" and "antalarmin" in either the medial or lateral parts of the orexin population. The student's t-test revealed elevated ppOX mRNA levels in the medial orexin portion in shock rats treated with vehicle ($t_{15} = 4.51$, $p < 0.001$; Figure 14a) as well as with antalarmin ($t_{18} = 2.51$, $p < 0.05$; Figure 14a). Similarly, an increase in ppOX mRNA level in the lateral part was found in shock rats with vehicle injection ($t_{15} = 2.20$, $p < 0.05$; Figure 14a) and antalarmin injection ($t_{18} = 2.18$, $p < 0.05$; Figure 14a) compared to their similarly treated nonshock ones.

In shock animals, the Pearson correlation test did not find any relationship between the total ppOX mRNA levels and the immobility expressed to the novel chamber on Day 9 in groups treated with vehicle ($R^2 = 0.38$, $p > 0.05$; Figure 14b) and antalarmin ($R^2 = 0.09$, $p > 0.05$; Figure 14b). However, the ppOX mRNA levels were found to be positively correlated with immobility response when vehicle-treated shock rats were placed in the shock chamber ($R^2 = 0.47$, $p < 0.05$; Figure 14b) at Days 10.

**Figure 14  Effect of the CRF receptor-1 antagonist antalarmin on ppOX mRNA levels**

Effects of CRF receptor-1 antagonist antalarmin on ppOX mRNA levels. (a) Antalarmin had no effect on ppOX mRNA levels.  (b) Antalarmin disturbed the positive correlation between ppOX mRNA levels and immobility percentage expressed by shock rats to the shock chamber at Day 10.  Values are mean $\pm$ SEM.  * $p < 0.05$, ** $p < 0.01$ compared to nonshock with vehicle injection;  # $p < 0.05$ compared to nonshock rats with antalarmin injection.
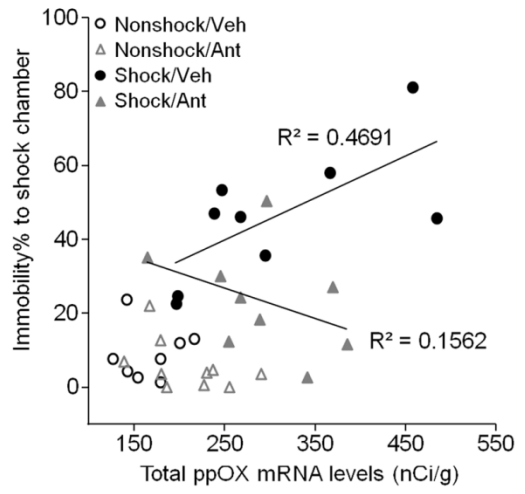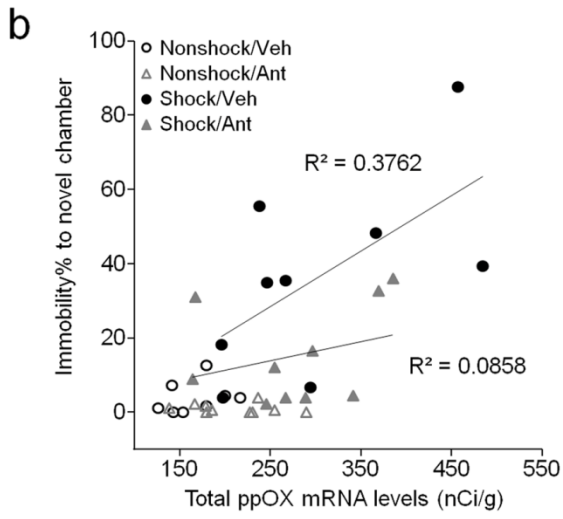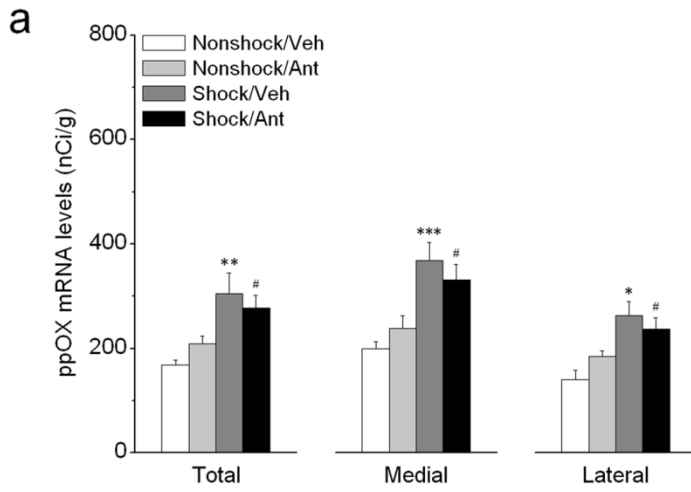
Pre-treatemnt with antalamin eliminated this positive correlation for the reason that no correlation was found in antalamin-treated shock rats ($R^2 = 0.16$, $p > 0.05$; Figure 14b). The Pearson correlation also showed no correlation between the levels of ppOX mRNA and the number of USV emitted to the shock context at Day 10 in shock rats ($R^2 = 0.19$, $p > 0.05$). Furthermore, no correlation was found in nonshock rats between the ppOX mRNA expression and their immobility response or USV calls displayed to the novel chamber or to the shock chamber.

In summary, the administration of the CRF receptor-1 antagonist antalarmin 30 min before exposing rats to footshocks was found to decrease the immobility response and USV calls expressed in shock rats when they were placed in the shock chamber at 2 and 10 days after the shock exposure. Additionally, antalarmin eliminated the positive correlation between the ppOX mRNA levels and the immobility response expressed to the shock chamber at Day 10 in shock rats, which indicates that blocking CRF receptor-1 at the time of the footshock plays a role in subsequent contextual fear expression and may mediate the shock induced-enhanced activity of the orexin system.

## 3.6.  Effects of Orexin Receptor Antagonist TCS-1102 on fear and Anxiety

Animals treated with the dual orexin receptor antagonist TCS-1102 were placed in an open field and the shock chamber two weeks after the footshock exposure to evaluate

whether the antagonism of orexin receptos can inhibit the expression of fear and anxiety. The two-way ANOVA revealed a main effect for "shock" ($F_{(1,67)}$ = 10.40, p < 0.01) and an interaction effect between "shock" and "TCS-1102" ($F_{(3,67)}$ = 4.56, p < 0.01) on immobility percentage in rats tested in the open field. The student's t-test showed an increase in immobility expressed in shock rats received vehicle ($t_{17}$ = 3.46, p < 0.01; Figure 15a) and 5 mg/kg TCS-1102 ($t_{16}$ = 2.98, p < 0.01; Figure 15a) compared to nonshock rats with similar treatment, but not in groups received 10 mg/kg ($t_{17}$ = 0.50, p > 0.05; Figure 15a) or 20 mg/kg TCS-1102 ($t_{16}$ = 1.06, p > 0.05; Figure 15a). The one-way ANOVA indicated a significant effect of "TCS-1102" on immobility expressed in shock rats ($F_{(3,34)}$ = 3.02, p < 0.05; Figure 15a). The post-hoc test showed that TCS-1102 at a dose of 10 mg/kg (p < 0.05) and 20 mg/kg (p < 0.05) attenuated the immobility expressed in shock rats exposed to a novel open field. No effect of TCS-1102 on immobility expressed in nonshock rats ($F_{(3,33)}$ = 1.88, p > 0.05; Figure 15a) was detected when they were placed in the open field.

The two-way ANOVA revealed a main effect for "shock" ($F_{(1,66)}$ = 67.859, p < 0.001) and an interaction effect between "shock" and "TCS-1102" ($F_{(3,66)}$ = 3.194, p < 0.05) on immobility response when rats were placed in the shock chamber. Shock rats showed increased immobility compared to nonshock rats when they were treated with vehicle ($t_{17}$ = 7.30, p < 0.001; Figure 15b), 5 mg/kg ($t_{16}$ = 6.22, p < 0.001; Figure 15b), and 10 mg/kg TCS-1102 ($t_{16}$ = 4.38, p < 0.001; Figure 15b), but not when they received 20 mg/kg TCS-1102 ($t_{16}$ = 1.24, p > 0.05; Figure 15b). The one-way ANOVA

**Figure 15  Effects of the orexin receptor antagonist TCS-1102 on fear and anxiety**

Effects of the orexin receptor antagonist TCS-1102 on expression of fear and anxiety when shock (Vehicle: n = 10; 5 mg/kg: n = 9; 10 mg/kg: n = 10; 20 mg/kg: n = 9) and nonshock rats (Vehicle: n = 10; 5 mg/kg: n = 9; 10 mg/kg: n = 9; 20 mg/kg: n = 9) were placed in an open field and the shock chamber.  (a)  TCS-1102 (10 and 20 mg/kg) decreased immobility response to the open field in shock rats.  (b) TCS-1102 (10 and 20 mg/kg) decreased immobility response to the shock chamber in shock rats.  (c)  TCS-1102 had no effect on locomotor activity when rats were placed in the open field.  (d) TCS-1102 (5, 10 and 20 mg/kg) decreased the latency for shock rats to enter the center of the open field.  Values are indicated as mean $\pm$ SEM.  Significant difference between groups with * $p < 0.05$; ** $p < 0.01$ compared to shock rats with vehicle injection.
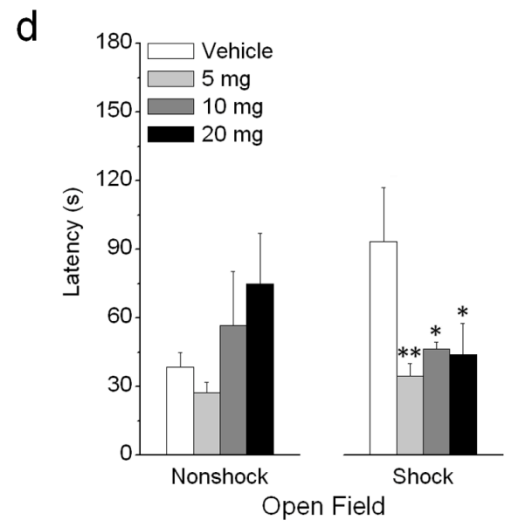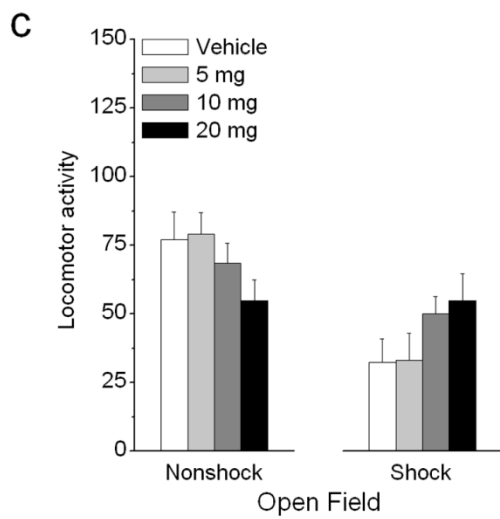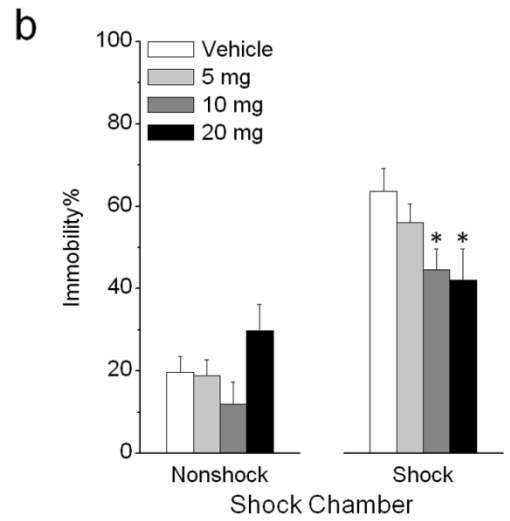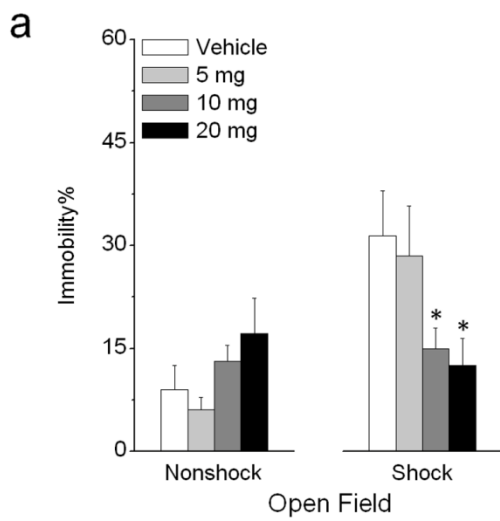
demonstrated a main effect for "TCS-1102" on immobility response in shock rats ($F_{(3,33)}$ = 3.08, p < 0.05; Figure 15b). The post-hoc analysis indicated that both the 10 mg/kg (p < 0.05) and 20 mg/kg (p < 0.05) doses decreased immobility expressed by shock rats when they were placed in the shock context. There was no effect of TCS-1102 on immobility expressed in nonshock rats ($F_{(3,33)}$ = 2.14, p > 0.05; Figure 15b) when they were exposed to the shock context.

For experiments involving the open field, the two-way ANOVA revealed a main effect for "shock" ($F_{(1,67)}$ = 20.253, p < 0.001) and an interaction effect between "shock" and "TCS-1102" ($F_{(3,67)}$ = 3.283, p < 0.05) for locomotor activity. The student's t-test detected a decrease in shock groups compared to nonshock groups when they received vehicle ($t_{18}$ = 3.40, p < 0.01; Figure 15c), or 5 mg/kg TCS-1102 ($t_{16}$ = 3.61, p < 0.01; Figure 15c). There was no difference between shock and nonshock rats when they received 10 mg/kg ($t_{17}$ = 1.87, p > 0.05; Figure 15c), or 20 mg/kg TCS-1102 ($t_{16}$ = 0.01, p > 0.05; Figure 15c), which indicates a potential drug effect. However, the one-way ANOVA detected no significant effect of "TCS-1102" on locomotor activity in shock ($F_{(3,34)}$ = 1.73, p > 0.05; Figure 15c) or nonshock rats ($F_{(3,33)}$ = 1.68, p > 0.05; Figure 15c).

The latency to enter to the center area of the open field was also calculated for comparing the effect of "shock" and "TCS-1102" on the levels of anxiety. The two-way ANOVA showed interaction effect between "shock" and "TCS-1102" on latency ($F_{(3,64)}$ = 2.74, p < 0.05; Figure 15d). Shock rats showed delayed latency compared to nonshock rats when they received vehicle treatment ($t_{17}$ = 2.13, p < 0.05; Figure 15d). But no difference between shock and nonshock rats was detected in groups that received 5 mg/kg ($t_{15}$ = 1.06, p > 0.05; Figure 15d), 10 mg/kg ($t_{16}$ = 0.43, p > 0.05; Figure 15d), or 20

mg/kg doses of TCS-1102 ($t_{16} = 1.20$, $p > 0.05$; Figure 15d).  The one-way ANOVA showed that there was an effect for TCS-1102 in shock ($F_{(3,32)} = 3.20$, $p < 0.05$, Figure 15d), and the post-hoc analysis indicated that 5 mg/kg ($p < 0.01$), 10 mg/kg ($p < 0.05$) or 20 mg/kg ($p < 0.05$) doses decreased the latency to enter to the center of the open field in shock rats.  No effect of TCS-1102 was found in nonshock rats ($F_{(3,32)} = 1.57$, $p > 0.05$; Figure 15d).

To summarize, the dual orexin receptor antagonist TCS-1102 attenuated the generalized and contextual fear responses in rats that received the shock exposure 14 days earlier.  TCS-1102 also decreased the latency to explore the center area of an open field which suggests that blocking of orexin receptors attenuates the levels of anxiety in shock rats.  Importantly, TCS-1102 had no effect on behaviors tested in nonshock rats when they were placed in an open field or the shock chamber.

## 3.7.  Effects of the Orexin Receptor Antagonist TCS-1102 on Avoidance and Escape

### 3.7.1.  HR and LR Subgroups of Shock Rats

In the previous studies, we found that shock rats that displayed a high level of acute fear to a novel tone (High Responders, HR) also showed high levels of anxiety in the social interaction and the defensive withdrawal tests compared to the shock rats that displayed a low level of acute fear response (Low Responsers, LR).  The present

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experiment examined the anxiolytic effect of a dual orexin receptor antagonist TCS-1102 when subgroups of shock rats and nonshock rats were examine at the ETM test. Similar as the previous experiment, shock rats were placed in a novel chamber and a novel tone was present to test their immobility responses. The student's t-test revealed an increase in immobility to a novel tone in shock rats compared to nonshock rats ($t_{88} = 11.86$, $p < 0.001$; Figure 16a). Immobility in shock rats ranged from 0 to 100% during the tone phase (Figure 16a). The immobility response to the novel tone was used to subdivide shock rats into LR (immobility < 40%) and HR (immobility > 60%) groups (Figure 16b) and the remainder of shock rats (immobility percentage ranging 40 - 60%) were omitted from further analysis. The group of HR showed more immobility compared to LR ($t_{55} = 3.70$, $p < 0.001$; Figure 16b).

### 3.7.2. Effects of TCS-1102 on Avoidance Behavior

To compare the "shock" effect, the one-way ANOVA was used to analyze the latency of each trial in rats with similar drug treatment. In vehicle-treated rats, there was a significant effect of "shock" on baseline latency ($F_{(2,26)} = 16.01$, $p < 0.001$; Figure 17a) and avoidance 1 latency ($F_{(2,26)} = 3.578$, $p < 0.05$; Figure 17a), but not on avoidance 2 latency ($F_{(2,26)} = 1.39$, $p > 0.05$). For the baseline trial, the post-hoc analysis revealed that LR ($p < 0.05$) and HR ($p < 0.001$) displayed increased baseline latency compared to nonshock rats, whereas HR showed a longer latency compared to LR ($p < 0.01$). For the avoidance 1 trial, HR exhibited longer latency compared to nonshock rats ($p < 0.05$) and LR ($p < 0.05$). In rats treated with 5 mg/kg TCS-1102,

**Figure 16  HR and LR Groups**

Acute fear response (immobility) to a novel tone one day after the shock exposure.  (a)

The individual immobility score and the mean immobility score for nonshock (n = 30)

and shock rats (n = 60).  Shock rats showed an increase in immobility responses.  (b)

Shock rats were subdivided into low responders (LR, immobility < 40%, n = 25) and high

responders (HR, immobility > 60%, n = 33) based on their immobility responses to the

novel tone.  The values in the histograms are mean ±SEM. *** p < 0.001.
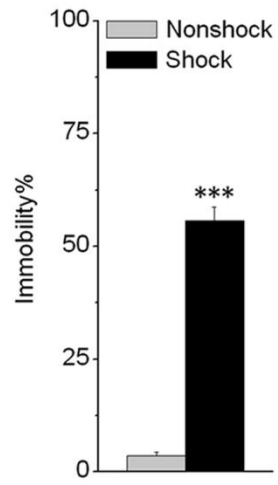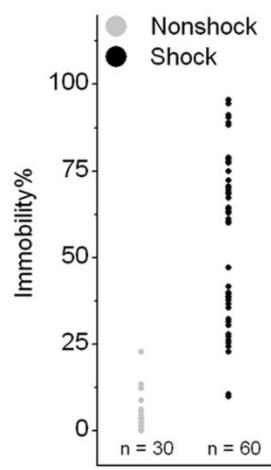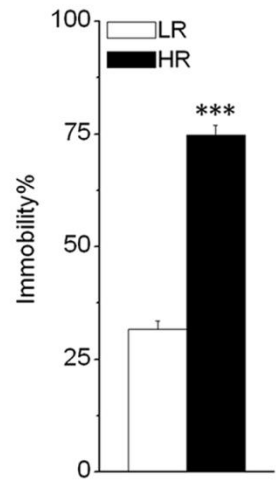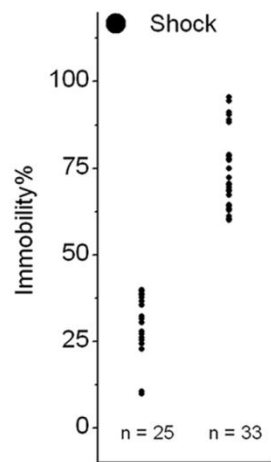
**Figure 17  Effects of the orexin receptor antagonist TCS-1102 on avoidance and escape**

Effects of shock and TCS-1102 on avoidance and escape behaviors in HR (Vehicle: n = 10; 5 mg/kg TCS-1102: n = 9; 10 mg/kg TCS-1102: n = 10) and nonshock rats (Vehicle: n = 10; 5 mg/kg TCS-1102: n = 9; 10 mg/kg TCS-1102: n = 9; 20 mg/kg: n = 9) measured in Elevated T-Maze (ETM).  (a) Baseline latency was increased in LR and HR and HR showed increased latency than LR.  10 mg/kg TCS-1102 decreased the latency specifically in the HR in baseline and all avoidance trials.  (b) There were no significant differences in latency between the different groups in escape trials and TSC-1102 had no effect on escape behavior.  The values in the histograms are mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$,*** $p < 0.001$ compared to HR group with vehicle injection.
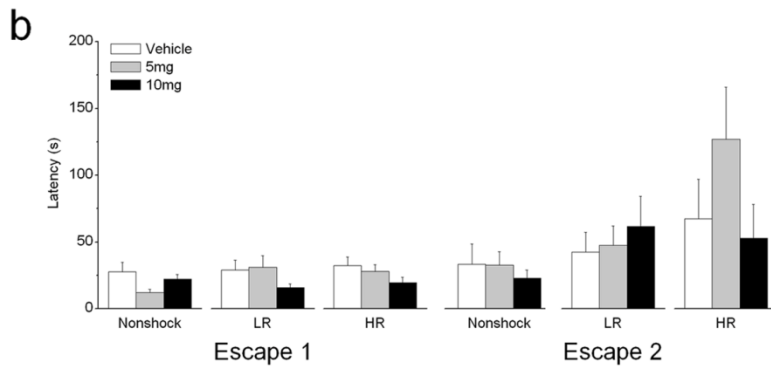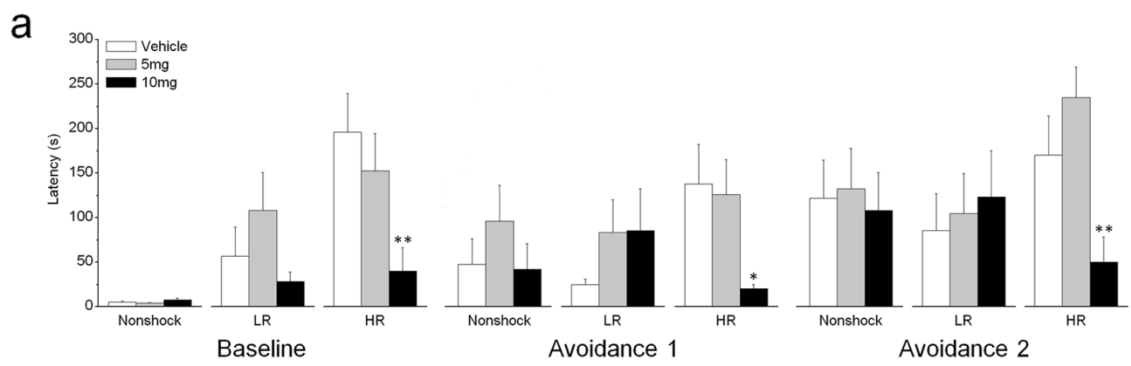
a "shock" effect was found in the baseline latency ($F_{(2,27)} = 19.44$, $p < 0.001$; Figure 17a), and post-hoc analysis showed  HR ($p < 0.001$) and LR ($p < 0.001$) displayed increased latency compared to nonshock rats.  No effect of "shock" was found in avoidance 1 ($F_{(2,27)} = 0.18$, $p > 0.05$) and  avoidance 2 ($F_{(2,27)} = 3.11$, $p > 0.05$).  Furthermore, no significance effect of "shock" was found in groups that received 10 mg/kg TCS-1102 (baseline: $F_{(2,25)} = 2.75$; $p > 0.05$; avoidance 1: $F_{(2,25)} = 0.51$; $p > 0.05$; avoidance 2: $F_{(2,25)} = 0.86$; $p > 0.05$).

In order to examine the effect of "TCS-1102" on avoidance latencies, a one-way ANOVA was used to analyze the latency at each trial at groups of nonshock rats, LR or HR.  The one-way ANOVA revealed that TCS-1102 had a significant effect on latency for the baseline ($F_{(2, 29)} = 4.801$; $p < 0.05$; Figure 17a), avoidance 1 ($F_{(2,29)} = 3.326$; $p < 0.05$; Figure 17a), and avoidance 2 ($F_{(2,29)} = 8.453$; $p < 0.01$; Figure 17a) trials displayed by HR.  The post-hoc analysis suggested that TCS-1102 at a dose of 10 mg/kg significant reduced the latency in the baseline ($p < 0.01$), avoidance 1($p < 0.05$) and avoidance 2 ($p < 0.01$) trials.  No significant effect of "TCS-1102" was found in LR (baseline: $F_{(2,22)} = 2.88$; $p > 0.05$; avoidance 1: $F_{(2,22)} = 0.86$; $p > 0.05$; avoidance 2: $F_{(2,22)} = 0.13$; $p > 0.05$) or nonshock rats (baseline: $F_{(2,27)} = 1.25$; $p > 0.05$; avoidance 1: $F_{(2,27)} = 0.60$; $p > 0.05$; avoidance 2: $F_{(2,27)} = 0.04$; $p > 0.05$).

The effect of "trial" was examined in groups of nonshock, LR and HR with vehicle injection.  The repeated one-way ANOVA showed an effect of "trial" on nonshock rats ($F_{(2,18)} = 22.018$, $p < 0.001$; Figure 17a) but not on LR ($F_{(2,16)} = 0.846$, $p > 0.05$) or HR ($F_{(2,22)} = 0.023$, $p > 0.05$).  The pair-wise comparison revealed an increase in avoidance 1 latency ($p < 0.01$) and avoidance 2 latency ($p < 0.001$) in nonshock rats

compared to baseline latency, and avoidance 2 latency is also longer compared to avoidance 1 latency ($p < 0.01$).

### 3.7.3. Effects of TCS-1102 on Escape Behavior

After the avoidance trials, rats were placed in the open arm of the ETM for two times to examine their escape behavior. The three-factor ANOVA revealed a significant main effect of "trial" on escape ($F_{(1,78)} = 6.999$; $p < 0.01$) but no effect of "shock" ($F_{(2,78)} = 0.993$; $p > 0.05$) or "TCS-1102" ($F_{(2,78)} = 0.395$; $p > 0.05$), nor was there interaction effect between "trial" and "shock" ($F_{(2,78)} = 0.825$; $p > 0.05$), "trial" and "TCS-1102" ($F_{(2,78)} = 2.417$; $p > 0.05$) or "shock" and "TCS-1102" ($F_{(4,78)} = 0.532$; $p > 0.05$). For the "trial" effect in vehicle-treated rats, escape latency was significantly increased in LR ($F_{(1,24)} = 4.756$, $p < 0.05$; Figure 17b) and HR ($F_{(1,31)} = 4.181$, $p < 0.05$; Figure 17b) groups, but not in nonshock rats ($F_{(1,29)} = 0.227$, $p > 0.05$; Figure 17b). The pair-wise comparison revealed that both of LR ($p < 0.05$) and HR ($p < 0.05$) showed increased latency in the second escape task compared to their first escape task.

In summary, rats that received footshock stress and showed a high level of fear to a novel tone one day after the footshock exposure (HR) displayed an increase in the baseline latency (a measure of anxiety) compared shock rats that displayed a low level of fear (LR) and nonshock rats. The dual orexin receptor antagonist TCS-1102 at dose of 10 mg/kg decreased the latency of baseline, avoidance 1 and avoidance 2 trials displayed

only in HR group, which suggested that the drug had anxiolytic effects that are specific

for HR.

# Chapter 4

# Discussion

## 4.1.  A Rat Model of PTSD

Experiments were done to test the hypothesis that acute generalized fear response predicts the long-term expression of anxiety.  The results demonstrated that exposure of rats to an acute episode of moderately intense footshock produced fear responses when rats are placed in a novel situation and exposed to unexpected novel tones when tested over approximately a four week period.  In addition to showing generalized fear responses, shock rats that exhibited a high level of fear to a novel tone (HR) one day after the shock exposure showed avoidance of open spaces and novel rats.  In contrast, shock rats that exhibited a low level of fear to a novel tone (LR) resembled nonshock rats in their responses to novel chambers, tones, open fields and novel rats.  The enduring fear and avoidance displayed by HR highlights the importance of individual differences for producing long-lasting behavioral changes in rats exposed to an acute footshock episode. These results are consistent with human studies indicating that the presence of an intense peritraumatic stress response may predict the subsequent development of PTSD (Cardena and Carlson 2011) and provide further validation of the rodent footshock as a useful model to study the mechanisms of PTSD.

Clinical evidence shows that individuals exposed to a life threatening and fear-inducing situation subsequently exhibit fear when confronted with reminders of the situation as well as novel situations not directly related to the trauma (Charney, Deutch et al. 1993; Stam 2007). While this is considered as a normal emotional response that eventually dissipates over time, the fear response associated with the trauma in some individuals does not extinguish and generalizes to many different situations (Charney, Deutch et al. 1993; Stam 2007). This can lead to significant distress as well as the adoption of an avoidance strategy to control the level of distress experienced (Charney, Deutch et al. 1993; Stam 2007). Experimental studies have consistently found that rodents exposed to intense electrical shock not only show a strong fear response when they are re-exposed to the shock apparatus, but also display fear-like responses when exposed to a novel environment or changes in background noise levels (van Dijken, Mos et al. 1992; Van Dijken, Tilders et al. 1992; Louvart, Maccari et al. 2005; Louvart, Maccari et al. 2006; Siegmund and Wotjak 2007; Siegmund and Wotjak 2007; Mikics, Baranyi et al. 2008; Daviu, Fuentes et al. 2010). The present experiment demonstrated that shock rats that showed the most intense fear 24 h after the footshock experience (i.e. HR) continued to show more fear to the shock context and novel chambers. While the experiments were not designed to determine the contribution of associative and non-associative learning mechanisms in fear expression, the enhanced fear response exhibited in shock rats exposed to novel chambers and tones is likely due to a combination of non-associative learning mechanism (fear sensitization) and associative learning mechanisms (fear generalization) (Kamprath and Wotjak 2004; Daviu, Fuentes et al. 2010). As data in the present experiment seem to indicate, sensitized and generalized fear would likely

habituate and extinguish more slowly in HR, an effect that is likely to become apparent with repeated testing.

The present studies also found that HR displayed avoidance of novel rats (social interaction test) and open areas (defensive withdrawal test) whereas LR showed a similar avoidance as nonshock rats. This is consistent with previous studies showing that rodents preexposed to electrical shock show enhanced avoidance in situations involving novel conspecifics, objects, or test areas (Bruijnzeel, Stam et al. 2001; Bruijnzeel, Stam et al. 2001; Louvart, Maccari et al. 2005; Mikics, Baranyi et al. 2008; Mikics, Toth et al. 2008). As such, it is reasonable to propose that the enhanced avoidance displayed by rodents previously exposed to shock results from an adaptive response where novel situations are fear-inducing and are approached with caution (Pamplona, Henes et al. 2011). Consequently, the behavioral response of shock rats is consistent with the hypothesis that exposure of rats to a single episode of moderately intense footshock produces an emotional state in which fear is more easily elicited. The fact that avoidance behaviors were only observed in HR highlights the importance of how individual rats react to the footshock experience as a factor that determines whether fear and avoidance will be expressed for a longer period of time. We also demonstrate for the first time that shock rats that show a lasting fear and avoidance to novel situations (HR) emit USV in the 20 - 30 kHz range. Since USV in this range in rats represent alarm calls associated with negative emotional states (Brudzynski 2001; Litvin, Blanchard et al. 2007), measurements of these calls may represent a useful addition to the tests used to established PTSD-like behavioral profiles in shock rats.

It is well-documented that individuals who experience a life-threatening trauma can subsequently exhibit fear and anxiety in situations not directly related to the trauma (Charney, Deutch et al. 1993; Stam 2007). One possible mechanism for this phenomenon is that the neural circuits that regulate fear become sensitized or more easily aroused (Charney, Deutch et al. 1993; Stam 2007) which can lead to fear and avoidance of situations not directly related to the trauma (Charney, Deutch et al. 1993). Based on animal research, it is also likely that fear generalization mechanisms are involved in the enhanced fear state associated with a trauma experience (Kamprath and Wotjak 2004; Daviu, Fuentes et al. 2010). It is apparent that factors associated with resilience and how individuals react to the trauma play an important role in determining if a trauma experience will lead to long-lasting psychological distress (Harvey and Bryant 2002; Shalev 2002). While it has been difficult to establish a causal link between the acute response to trauma and the subsequent development of PTSD in human studies (Harvey and Bryant 2002; Shalev 2002), the idea has intuitive appeal and some clinical support (Cardena and Carlson 2011). The present experiment makes use of the advantages provided by an animal model to determine if the intensity of the acute fear generalization response is related to subsequent changes in emotional behaviors. These advantages include a homogenous population (strain, age and sex) with identical life experience (pre-trauma housing and handling, standardized trauma, and post-trauma conditions) along with assessment of behavioral reaction to standardized tests that do not rely on subjective reports of emotions. Using this approach, we find that the behavioral pattern observed in rats exposed to footshock supports the view that the peritraumatic fear response to a novel situation is a good predictor of a lasting fear and avoidance. We interpret this

enhanced fear generalized state as analogous to the hyperarousal symptoms seen in humans exposed to trauma, which have been found to be a good predictor of future psychological distress (Weems, Saltzman et al. 2003; Schell, Marshall et al. 2004; Thompson, Vasterling et al. 2004; Feuer, Nishith et al. 2005; Marshall, Schell et al. 2006; Solomon, Horesh et al. 2009). As such, these results provide support for the view that an early display of pronounced fear following trauma may be associated with long-lasting changes in how the brain processes novel stimuli and situations in a direction which promotes fear and avoidance. The results also underscore the importance of individual differences in the peritraumatic fear response and further validate the rodent footshock model to study the neural mechanism associated with the hyperarousal and avoidance symptoms of PTSD.

## 4.2. Fear Extinction Experiment

Experiments were done to assess the contribution of fear generalization and fear sensitization mechanisms to the fear displayed when shock rats are placed in a novel environment. The results showed that fear extinction (repeated exposure of rats in the shock chamber without receiving footshocks) attenuated the immobility displayed by shock rats when they were placed in a novel chamber. However, the shock rats exposed to the fear extinction procedure showed an increase in immobility to a novel tone compared to shock rats that had not receive the extinction procedure. This indicates that

fear extinction has a differential effect on the fear response to the novel chamber compared to the novel tone. These results suggest that fear generalization as well as fear sensitization occurs when rats are exposed to a brief episode of moderately intense footshock.

Previous research has reported that rats that received footshocks displayed enhanced immobility (or freezing) to a novel chamber as well as a novel auditory tone (van Dijken, Mos et al. 1992; Louvart, Maccari et al. 2005; Louvart, Maccari et al. 2006; Siegmund and Wotjak 2007; Mikics, Baranyi et al. 2008; Ito, Pan et al. 2009; Daviu, Fuentes et al. 2010). The fear response to a novel chamber has been hypothesized to result from a combination of fear generalization (a novel chamber triggers fear memories that is related to the shock chamber; i.e. an associative learning mechanism) and fear sensitization mechanisms (the fear systems of the brain are more sensitive to potentially threatening environment or stimuli; i.e. a non-associative learning mechanism) (Kamprath and Wotjak 2004; Daviu, Fuentes et al. 2010). The present results show that repeated exposure of shock rats to the chamber in which they received footshocks decreases the fear to the shock chamber as shown in other studies in which fear extinction eliminates contextual fear expression (Myers and Davis 2002; Golub, Mauch et al. 2009). In these animals, fear to a novel chamber is also attenuated to the level of that expressed in nonshock rats which indicates that fear to a novel chamber is mainly mediated through an associative fear learning mechanism. This is consistent with other studies that demonstrated that fear extinction attenuates generalized fear (Golub, Mauch et al. 2009). On the other hand, the fear extinction procedure also produced a decrease in the immobility response expressed in shock rats when the novel tone is present. However,

the shock/extinction rats still showed increased immobility compared to the nonshock rats in the presence of the novel tone, which indicates a sensitized fear response in those rats. As a whole, the results of the extinction experiments indicate that the fear response to a novel tone is mediated by both associative and non-associative learning mechanisms.

Previous work has shown that exposure of rodents to footshocks produced long-lasting hypoactivity when these rodents are placed in novel environments (van Dijken, Mos et al. 1992; Van Dijken, Van der Heyden et al. 1992; Daviu, Fuentes et al. 2010). This immobility is interpreted as evidence of enhanced anxiety as a result of footshocks (van Dijken, Mos et al. 1992). The present results of thesis experiments show that fear extinction significantly enhances locomotor activity in shock rats when they were exposed to a novel chamber and a novel tone. However, the shock/extinction rats still showed decreased locomotor activity compared to nonshock rats.

Exposure therapy which is used to treat people with PTSD has been found to effectively manage some of the symptoms of the disorder (Marks, Lovell et al. 1998; Foa, Dancu et al. 1999; Rauch, Eftekhari et al. 2012; van Minnen, Harned et al. 2012). The present study used a footshock model in rats to demonstrates that fear extinction attenuates generalized fear. This suggests that exposure therapy may not be useful for eliminating the anxiety that may occurs through fear sensitization mechanisms.

## 4.3.  Effects of Footshock on ppOX mRNA Levels

The ppOX mRNA levels in the hypothalamus were evaluated in rats exposed to moderately intense footshocks at 6 and 14 days after the shock exposure in order to test the hypothesis that footshock stress produces a long-term upregulation in the orexin system.  The results demonstrated that the expression of ppOX mRNA levels was elevated in rats at 6 days and 14 days post-shock.  At the later time point, ppOX mRNA levels were found to be positively correlated with immobility displayed by shock rats when they were placed in the shock chamber (contextual fear).  The results suggest that the levels of ppOX mRNA in orexin neurons are involved in fear responses in rats that received a single episode of footshocks.

Previous studies have provided evidence for a short-term increase in orexin neuron activity following acute episodes of stress.  For example, an increase in ppOX mRNA levels in response to one hour of immobilization stress was reported for rats immediately sacrificed after the stress episode (Ida, Nakahara et al. 2000).  In addition, a larger number of cFos expressing orexin neurons were found in rodents exposed to either a series of footshocks, novelty stress, chronic mild stress, or a chamber where shock had been previously given over several days (Zhu, Onaka et al. 2002; Espana, Valentino et al. 2003; Sakamoto, Yamada et al. 2004; Furlong, Vianna et al. 2009; Rachalski, Alexandre et al. 2009; Nollet, Gaillard et al. 2011).  The present experiment extends these findings by demonstrating that exposure of rats to a single episode of footshocks produced a lasting increase in the levels of ppOX mRNA up to two weeks after the shock exposure. The increase in ppOX mRNA appears to reflect a change in the baseline activity of orexin

neurons because the brain samples were taken 4 days after the last behavioral test.  Thus, this experiment suggests that a single episode of moderately intense footshocks produce increased basal levels of ppOX mRNA.

The experiment also showed that the levels of ppOX mRNA were increased in both the medial and lateral population of orexin neurons in shock rats at both the early and late time points.  It has been proposed that the medial orexin population may regulate arousal levels associated with stress and negative emotions while the lateral population may be preferentially involved in food and drug reward (Harris, Wimmer et al. 2005; Furlong, Vianna et al. 2009).  The consequence of a shock-induced increase in orexin levels in the lateral as well as the medial orexin population is not known but could represent a mechanism by which general behavioral arousal is increased following the shock experience.

The recording of immobility (or freezing) is a commonly used behavioral index to quantify the expression of fear (Fanselow 1980; Blanchard, Yudko et al. 1993; Antoniadis and McDonald 1999).  The present results showed that shock rats displayed increased immobility to the shock chamber as well as to a novel chamber, which is consistent with previous results that have demonstrated rodents exposed to intense electrical shock not only show a strong contextual fear response when they are re-exposed to the shock apparatus, but also display generalized fear-like responses when exposed to novel environment (van Dijken, Mos et al. 1992; Louvart, Maccari et al. 2005; Louvart, Maccari et al. 2006; Siegmund and Wotjak 2007; Mikics, Baranyi et al. 2008; Daviu, Fuentes et al. 2010).  The increased ppOX mRNA level was found to be positively correlated with the amount of immobility expressed by shock rats placed in the shock

chamber at late time point. A small trend was observed in correlations between ppOX mRNA levels and immobility expressed to a novel chamber at late time point, but no significance was found. These results suggest that the levels of ppOX mRNA contributes to the expression of contextual fear. No correlation was found in the early expression of ppOX mRNA and immobility, which provided further evidence that the function of orexin neurons play a role in promoting the long-lasting effect of footshock stress on fear expression.

## 4.4. Changes in the Activity of Orexin Neurons During Expression of Fear

In this experiment, we tested the hypothesis that orexin neurons are activated by conditions associated with an aversive event (e.g. novelty stress or conditioned responses). This study reported an increase in the number of orexin neurons that also express cFos protein when both shock and nonshock rats were placed in a novel environment or the shock context 14 days after the shock event. However, no difference between nonshock and shock rats was found in the number of double-labeled neurons when they are placed in the home cage, novel chamber or shock chamber. The results are consistent with the view that orexin neurons are activated in conditions involving arousal (Mileykovskiy, Kiyashchenko et al. 2005) but that these neurons do not respond more strongly to a situation previously associated with an aversive event (Zhu, Onaka et al. 2002).

This experiment also demonstrated that an increased number of orexin neurons in the medial population expressed cFos protein when rats were placed in the novel and shock chamber. In contrast, the lateral orexin population did not showed an elevation of cFos protein. This results is consistent with the view that the medial population regulates stressful emotional arousal while the lateral population is involved in food and drug reward (Harris, Wimmer et al. 2005; Furlong, Vianna et al. 2009). Consequently, it appears that an elevated level of ppOX mRNA in shock rats is not due to an activation of orexin- expressing neurons produced by post-shock exposure of shock rats to stressful or fear-inducing situation. These results suggest that the experience of the footshock may cause a stress-mediated activation on orexin-expressing neurons that is maintained long after the shock experience. This may result from the excitatory action of the stress hormone corticotropin releasing factor (CRF) on orexin neurons (Winsky-Sommerer, Boutrel et al. 2005).

## 4.5. Effects of the CRF Receptor-1 Antagonist Antalarmin on Fear and ppOX mRNA Levels

Studies have shown that orexin neurons may be activated through a CRF mechanism in response to stress (Sakamoto, Yamada et al. 2004; Winsky-Sommerer, Yamanaka et al. 2004; Winsky-Sommerer, Boutrel et al. 2005). The present experiments were done to determine if injections of the CRF receptor-1 antagonist antalarmin given

30 min before exposing rats to a series of moderately intense footshocks attenuated fear and increases in ppOX mRNA levels produced by footshocks.  The results demonstrated that pre-shock injections of antalamin decreased immobility and ultrasonic vocalization (USV) emission in shock rats placed in the shock chamber at 2 and 10 days after the shock episode.  In addition, the pre-shock injections of antalarmin eliminated the positive correlation between ppOX mRNA levels and immobility response displayed to the shock chamber in shock rats.  These results indicate that activation of CRF receptor-1 at the time of the footshocks plays a role in subsequent contextual fear expression which may be mediated in part by enhanced activity of the orexin system.

Rodents produce USV in addition to immobility when they are exposed to footshock stress or a fearful situation (Swiergiel, Zhou et al. 2007; Chen, Li et al. 2012). USV in the range of 22 kHz represent defensive and alarm calls associated with negative emotional states in rats including fear (Blanchard, Yudko et al. 1993; Antoniadis and McDonald 1999; Brudzynski 2001; Wohr, Borta et al. 2005; Litvin, Blanchard et al. 2007).  In the present experiment, antalarmin given 30 min before the shock exposure did not have any effect on the immobility or the number of USV emitted during shock delivery.  The immediate effect of shock on these two indices of fear may be not regulated through the CRF receptor-1.  These results are consistent with other studies that showed no effect of pre-injection of nonselective CRF receptor antagonists on USV emission at the time of the footshocks (Kikusui, Takeuchi et al. 2000).  This lack of an effect of antalarmin on immobility and USV at the time of shock suggests that the intensity of the emotional reaction to the footshock episode was similar between antalarmin- and vehicle-treated rats.

A series of studies have already demonstrated that rodents exposed to intense electrical footshock not only show a strong contextual fear response when they are re-exposed to the shock apparatus, but also display generalized fear-like responses when exposed to a novel environment (van Dijken, Mos et al. 1992; Louvart, Maccari et al. 2005; Louvart, Maccari et al. 2006; Siegmund and Wotjak 2007; Mikics, Baranyi et al. 2008; Daviu, Fuentes et al. 2010). The presented results showed that pre-shock treatment with antalarmin had no effect on immobility when rats were exposed to a novel chamber or a novel tone several days later, indicating that the CRF receptor-1 is not involved in the generalized fear response. However, an effect of the drug on locomotor activity was found when nonshock rats were exposed to the novel chamber at Day 9 post-shock. The acute effect of CRF on locomotor activity in nonshock rats has been previously reported in studies in which a low dose of CRF produce increased locomotor activity (Sutton, Koob et al. 1982; Lee, Tang et al. 1987) while a high dose of CRF produce decreased locomotor activity when rodents are placed in a familiar environment (Imaki, Shibasaki et al. 1987; Lee, Tang et al. 1987). A novel chamber represents a mild stressor which can provoke a stress reaction and cause increased levels of CRF. The resented results suggest that blocking of CRF receptor-1 has a long-term effect on locomotor activity when rats are placed at a novel chamber. Since this effect was not seen in shock rats, it suggests that shock animals may have different mechanisms to produce the suppression of locomotor activity in response to a novel environment.

CRF has been shown to be involved in conditioned fear and anxiety (Swerdlow, Britton et al. 1989; Takahashi 2001; Skorzewska, Bidzinski et al. 2008). The present results demonstrated that pre-shock injections of antalarmin resulted in an attenuation of

the immobility and the number of USV calls observed in shock rats placed in the shock context tested at 2 and 10 days after the shock exposure. These results are consistent with previous studies showing that treatment of shock rats with CRF antagonists attenuated the induction of contextual fear. For example, CRF receptor-1 antagonists treated before or after shock exposure (within 24 hrs) decreased the immobility (Deak, Nguyen et al. 1999; Hikichi, Akiyoshi et al. 2000), and the number of USV emission (Kikusui, Takeuchi et al. 2000) displayed to the conditions where they received aversive stimuli. The present results highlight the importance of CRF receptor-1 on the long-term expression of contextual fear up to 10 days after the shock episode. We also found that shock rats that were pre-treated with antalarmin showed increased immobility compared to nonshock rats while showing similar levels of USV emission. This is consistent with a number of studies that have reported that activation of the CRF receptor-1 at the time of the footshock experience is an important determinant of the intensity of the conditioned fear response that is expressed at a later time.

Previous work has shown that exposure of rats to footshocks produces a lasting increase in ppOX mRNA levels and that ppOX mRNA levels are correlated with the amount of immobility displayed when these rats are placed back in the conditioning chamber. However, the mechanisms associated with footshock-induced upregulation of ppOX mRNA are not known. One possibility is that stress-induced release of CRF at the time of the footshocks leads to activation of CRF receptor-1 receptor on orexin neurons. For example, it has been reported that CRF depolarizes orexin neurons through a CRF receptor-1 mechanism and that activation of orexin-expressing neurons by footshock and restraint stress is impaired in CRF receptor-1 knockout mice (Winsky-Sommerer,

Yamanaka et al. 2004).  We tested this hypothesis by comparing the levels of ppOX mRNA 14 day after receiving footshocks in rats that had received either antalarmin or vehicle 30 min before the footschock exposure.  As shown in previous studies, shock rats displayed enhanced levels of ppOX mRNA relative to nonshock rats.  There was slight decrease in ppOX mRNA in shock rats treated with antalarmin compared to non-treated shock rats but the differences were not statistically significant.  As previously reported, there was a correlation between immobility and ppOX mRNA levels in vehicle-treated shock rats.  More importantly, this correlation was absent in shock rats that had been treated with antalarmin.  It appears that the correlation analysis provided a more sensitive measure of the effect of antalarmin on ppOX mRNA activity.  These results suggest that blocking of CRF receptor-1 at the time of the footshock attenuates the long-term upregulation of the orexin system in a way which interfered with the full expression of conditioned fear.  However, it is not known whether these effects were due to the activation of orexin-expressing neurons by CRF receptor-1.  For instance, CRF receptor-1 is expressed on neurons located in a number of neuronal systems in the brain known to mediate some of the endocrine, physiological and behavioral responses to stress (Heinrichs, Lapsansky et al. 1997; Bale and Vale 2004).  It is possible that the effect of pre-shock injections of antalarmin were mediated indirectly by the action of this CRF receptor-1 antagonist on other neurons in the brain that in turn modulate the activity of orexin neurons.

Previous studies have provided evidence in favor of a role for CRF receptor-1 in the initiation of negative emotional states associated with stress (Heinrichs, Lapsansky et al. 1997; Bale and Vale 2004).  For instance, blocking of CRF receptor-1 in mice exposed

to predator stress was shown to attenuate the acoustic startle response several days later (Adamec, Fougere et al. 2010). The present experiment provides support for the hypothesis that activation of CRF receptor-1 at the time of footshock contributes to subsequent fear responses possibly through an orexin-mediated mechanism. In my other experiments, the levels of ppOX mRNA at 14 days after the shock exposure were found to be correlated only with the expression of contextual fear expressed at late point, whereas the ppOX levels at 6 days post-shock was not correlated with contextual fear expression at that early time point. A possible explanation is that orexins are more likely contributing to the long-term expression of fear. The CRF receptor-1 antagonist given before the shock episode potentially interfered with the increase in orexins levels and contextual fear expression.

## 4.6. Effects of Orexin Receptor Antagonist TCS-1102 on Fear and Anxiety

The previous experiments have shown that ppOX mRNA levels were increased in rats that received a single episode of footshocks, and that the ppOX mRNA levels were correlated with fear expressed to the shock chamber. The present experiment was done to test the hypothesis that orexin receptors are involved with fear expression. The results demonstrated that the dual orexin receptor antagonist TCS-1102 attenuated the expression of immobility to an open field (generalized fear), the shock chamber

(contextual fear), as well as latency to explore the center of the open field (a measure of anxiety) 14 days after the shock exposure. The results indicate that orexin receptor antagonists regulate the expression of fear and anxiety produced by the footshock exposure.

The results of this experiment demonstrated that TCS-1102 attenuated the immobility expressed to an open field and the shock chamber in shock rats tested 14 days after the footshock exposure. A role for orexins in conditioned fear expression was also provided by a recent study demonstrating that almorexant reduced fear-induced startle in rats conditioned the day before to tones paired with mild footshocks (Steiner, Lecourt et al. 2012). Additionally, another study reported that oral administration of the dual orexin receptor antagonist almorexant attenuated the increases in heart rate and blood pressure produced by contextual fear (Furlong, Vianna et al. 2009). In contrast, a previous study reported that oral administration of a different dual orexin receptor antagonist almorexant did not have significant effect on contextual immobility (Furlong, Vianna et al. 2009). Potential differences that might account for the discrepant findings are the route of administration (i.p. vs. oral), footshock protocols used (single one day exposure vs. repeated over several days), intensity of the footshocks (2 s of 1.5 mA vs. 1 s of 1 mA) and length of time after last footshock exposure (14 days vs. a few days). In addition, TCS-1102 has a slightly higher proportional affinity to the $OX_2$ receptor ($IC_{50}$ are 17 nM for the $OX_1$ receptor and 4 nM for the $OX_2$ receptor) (Bergman, Roecker et al. 2008) compared to almorexant which has a slightly higher affinity to the $OX_1$ receptor ($IC_{50}$ are 13 nM for the $OX_1$ receptor and 8 nM for the $OX_2$ receptor) (Brisbare-Roch, Dingemanse et al. 2007). The stronger affinity of TCS-1102 to the $OX_2$ receptor may be of

importance because the $OX_2$ receptor has been linked to arousal of negative emotions involving fear, anxiety and avoidance (Li, Li et al. 2010; Li, Wang et al. 2011).

Rats have an innate motivation to explore a novel environment like the open field while the center of the open field represents an area that produces some level of avoidance in anxious rodents (Ramos and Mormede 1998). TCS-1102 decreased the latency to enter the centre of the open field in shock rats indicating that the drug has an anxiolytic effect. This observation is in line with previous studies reporting injection of orexins in the brain produces anxiety-like effects (Suzuki, Beuckmann et al. 2005; Li, Li et al. 2009; Li, Li et al. 2010; Li, Li et al. 2010; Heydendael, Sharma et al. 2011; Johnson, Samuels et al. 2012; Lungwitz, Molosh et al. 2012) and that central administration of orexin receptor antagonists has anxiolytic effects (Li, Li et al. 2010; Heydendael, Sharma et al. 2011; Johnson, Samuels et al. 2012). It is of note that the low dose of TCS-1102 (5 mg/kg) had anxiolytic effects in the open field but had no effect on the amount of immobility expressed by shock rats placed in the open field. This suggests that orexin receptors may play a more prominent role in anxiety than they do in conditioned fear expression.

## 4.7. Effects of the Orexin Receptor Antagonist TCS-1102 on Avoidance and Escape

Previous research showed that individual differences in the acute reaction (24 hours) to moderately intense footshocks accounts for the variability in anxiety-like behaviors displayed later on by shocked rats (Rau, DeCola et al. 2005; Chen, Li et al. 2012). The present experiment evaluated the effect of a brief footshock exposure and a dual orexin receptor antagonist (TCS-1102) on inhibitory avoidance and one-way escape response in rats tested on the ETM. Shock rats were separated into high responders (HR) that expressed more than 60 % immobility to a novel tone one day after the shock exposure, and low responders (LR) that expressed less than 40% immobility to a novel tone. The results showed HR displayed more avoidance compared to nonshock rats and LR, and TCS-1102 attenuated the avoidance behaviors only in HR, indicating that the drug has an anxiolytic effect specifically in animals with high levels of anxiety.

The present experiment demonstrated that HR displayed increased baseline latency compared to nonshock rats and LR in the ETM test at 14 days after the shock exposure. The ETM is generally considered to be a test of unlearned anxiety because the avoidance of the open arm by rodents is hypothesized to be due to fear of open spaces or height and a preference for the safety of the closed arm (Rodgers, Cao et al. 1997; Wall and Messier 2001; Carobrez and Bertoglio 2005). The results showed an increase in latency in the baseline and avoidance 1 trials displayed by LR and HR, which is consistent with previous studies that reported that rats submitted to footshock stress

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expressed more avoidance behavior in social interaction, defensive withdrawal, light-dark test, and the elevated plus-maze (van Dijken, Mos et al. 1992; Bruijnzeel, Stam et al. 2001; Bruijnzeel, Stam et al. 2001; Louvart, Maccari et al. 2005; Mikics, Baranyi et al. 2008; Mikics, Toth et al. 2008). The presented results also showed that HR took longer to exit the safety of the closed arm during the baseline and avoidance 1 phase of the test compared to LR and nonshock rats, indicating that HR expressed a higher level of anxiety compared to other rats. This is similar to the results of previous study which demonstrated that the acute immobility to a novel tone is predictive of anxiety tested in the social interaction and defensive withdrawal tests (Chen, Li et al. 2012). The results suggest that individual difference in response to peritraumatic stress is important in the development of anxiety.

The results of the ETM test also demonstrated that the latency to enter an open arm of the ETM apparatus is increased over the three trials in nonshock rats, indicating the acquisition of inhibitory avoidance. This type of avoidance learning was not seen in LR and HR, possibly because of the high rates of avoidance already present in shock rats. The ETM is similar to the elevated plus maze in that both tests evaluate anxiety levels of rodents by assessing how much time rodents avoid the fear-inducing open and elevated arms. The advantage of the ETM as a measurement of anxiety-like and avoidance behaviors is it can evaluate inhibitory avoidance (increased latency to leave the closed arm with successive trials) and one-way escape (latency to escape from the open arms) in the same animals (Viana, Tomaz et al. 1994; Zangrossi and Graeff 1997; Graeff, Netto et al. 1998). Another advantage of the ETM is that this test can assess escape behavior which may be indicative of helplessness (Viana, Tomaz et al. 1994; Zangrossi and Graeff

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1997).  In the one-way escape task, a trial effect was detected in LR and HR, but not in nonshock rats, indicating that the shock exposure impaired escape responses from the open arms of the ETM, which is consistent with other studies reporting that shock rats present poor escape performance in a subsequent footshock test (Overmier and Seligman 1967) or the ETM test (de Paula Soares, Vicente et al. 2011).

At last, the present research also showed the dual orexin receptor antagonist TCS-1102 in a dose of 10 mg/kg decreased the latency in the baseline, avoidance 1, and avoidance 2 phases of the ETM test only in HR.  Orexin receptor antagonists are currently being developed for their potential use in treating insomnia (Coleman and Renger 2010).  The dual orexin receptor antagonist TCS-1102 was also reported to promote sleep (Bergman, Roecker et al. 2008; Winrow, Tanis et al. 2010).  As discussed earlier, my experiments showed that TCS-1102 in a dose range of 5 mg to 20 mg had an anxiolytic effect on fear and anxiety behaviors expressed by shock rats tested in an open field or the shock chamber.  The ETM study also showed that TCS-1102 at a dose of 10 mg decreased the latency to enter to open arms of the ETM in a subgroup of shock rats (HR).  This indicates a specific anxiolytic effect of the drug on highly anxious animals. The anxiolytic effect of orexin antagonist have also been reported in shock and nonshock rats tested on the elevated plus-maze (Li, Li et al. 2010; Heydendael, Sharma et al. 2011) as well as in rats in a $CO_2$ induced anxiety state (Johnson, Samuels et al. 2012). Additionally, Johnson and colleagues have showed that systemic injections of an $OX_1$ receptor antagonist attenuated sodium lactate provoked social anxiety, defensive burying, and cardioexcitatory responses in panic-prone rats (Johnson, Truitt et al. 2010; Johnson, Molosh et al. 2012).

The ETM test has been shown to be particularly effective at screening anxiolytic drugs. Pharmacological studies have showed that treatment with classic anxiolytic agents such as benzodiazepine is able to reduce avoidance latencies without affect the escape behaviors in the ETM test (Graeff, Viana et al. 1993; Viana, Tomaz et al. 1994; Pinheiro, Del-Ben et al. 2008). In fact, studies show that anti-panic drugs increase the escape latencies without affect the avoidance response in the ETM test (Graeff, Netto et al. 1998; Zangrossi, Viana et al. 2001). As such, it is hypothesized that the orexin receptor antagonist may have an anti-panic effect on the escape behavior on the ETM. However, our result showed that TCS-1102 had no effect on one-way escape behavior, which suggested that the drug had no anti-panic effect on the behavior response of shock rats in the ETM test. Consequently, selective effect of the orexin receptor antagonist TCS-1102 on the avoidance component of the ETM test is similar to the results of pharmacological studies reporting the ability of anxiolytic agents like benzodiazepine to reduce avoidance latencies without affect the escape behaviors on the ETM. Therefore, the result provided the evidence that the orexin receptor antagonists may prevent avoidance-like behaviors selectively in animals with a high level of anxiety.

## 4.8. Summary: Roles of Orexins in PTSD

In this thesis, a footshock model of PTSD was improved by the grouping of shock rats into LR and HR based on their fear response to a novel tone one day after the shock exposure. The HR, which show a high level of acute fear to the tone showed an increase in anxiety-like behaviors several weeks after the shock exposure. In contrast, LR, which showed a low level of acute fear to the tone, had levels of anxiety similar to nonshock rats. We can also conclude from the extinction experiments that the fear response to novel tones is due to a combination of fear generalization and sensitization mechanisms. The enhanced acute fear response to a novel tone in shock rats may be analogous to some of the hyperarousal symptoms seen in human exposed to trauma. Thus, neuronal mechanisms associated with arousal may be involved in the development of PTSD.

Orexins are important for maintaining wakefulness and modulating arousal levels during goal-directed behavior (Taheri and Hafizi 2002; Sakurai 2007; Boutrel, Cannella et al. 2010). In addition, it has been proposed that the orexin system also becomes activated following exposure to conditions involving a strong fear or stress. The evolutionary benefit of such a response would be to increase vigilance and defensive behaviors so that organisms have a better chance of survival. Consistent with this hypothesis, we found that exposure of rats to a moderately intense episode of footshocks increased the levels of ppOX mRNA and that the levels of ppOX mRNA were correlated with the fear expressed by shock rats. Consequently, it is possible that some of the symptoms associated with the clinical diagnosis of PTSD may also result from enhanced activity of orexin neurons.

In rats that received footshocks, the elevated ppOX mRNA levels were found to be correlated with immobility expressed to the shock context. However, it is not clear if there is a causal relationship between ppOX mRNA levels and intensity of the contextual fear response. It is well-documented that orexin neurons become more active during or immediately after periods involving some form of behavioral arousal (Estabrooke, McCarthy et al. 2001; Espana, Valentino et al. 2003; Harris, Wimmer et al. 2005; Mileykovskiy, Kiyashchenko et al. 2005; Dayas, McGranahan et al. 2008) and that rodents exposed to moderately intense footshocks show a high level of generalized fear (Baldi, Lorenzini et al. 2004; Chen, Li et al. 2012; Sauerhofer, Pamplona et al. 2012). Consequently, increases in orexin activity could be the consequence of generalized fear and not a mediator of the fear state. However, this does not appear to be the case since the number of orexin neurons activated in shock rats exposed to a novel environment or the shock chamber was not different than the number of activated orexin neurons observed in nonshock rats placed in the same chamber. The possibility that orexins contribute to feat is supported by other studies that provide evidence for the role of the orexin system in contextual fear expression. First, the dual orexin receptor antagonist almorexant was shown to reduce fear-induced startle in rats conditioned with mild footshocks (Steiner, Lecourt et al. 2012). Second, almorexant was reported to attenuate the increases in heart rate and blood pressure produced by contextual fear (Furlong, Vianna et al. 2009). Third, the dual orexin receptor antagonist TCS-1102 was reported in this thesis to attenuate contextual fear 14 days after exposure to footshocks.

A number of studies have reported that injections of orexins in the brain produce fear and anxiety (Suzuki, Beuckmann et al. 2005; Li, Li et al. 2009; Li, Li et al. 2010; Li,

Li et al. 2010; Heydendael, Sharma et al. 2011; Johnson, Samuels et al. 2012; Lungwitz, Molosh et al. 2012) whereas administrations of orexin receptor antagonists have anxiolytic effects (Johnson, Truitt et al. 2010; Li, Li et al. 2010; Heydendael, Sharma et al. 2011). Previous research shows that individual differences in the acute reaction (24 hours) to moderately intense footshocks accounts for the variability in anxiety-like behaviors displayed later on by shocked rats (Rau, DeCola et al. 2005; Chen, Li et al. 2012). This thesis also discussed the role of orexin in expression in anxiety in a rat model of PTSD by separating shock rats into HR and LR. A study using quantitative polymerase chain reaction analysis of hypothalamic samples collected from HR, LR and nonshock groups showed that HR had higher levels of ppOX mRNA compared to LR and nonshock rats (Chen, Wang et al. 2013). Additionally, the thesis reported that the dual orexin receptor antagonist TCS-1102 at 10 mg/kg dose attenuated avoidance in HR tested in the ETM. We propose that the HR represent the subjects that resemble the subgroup of humans that go on to develop PTSD after a traumatizing experience. The fact that TCS-1102 had anxiolytic effects that were specific to the HR points to the possibility that orexin antagonists could have selective anxiolytic effects in people with PTSD. Consequently, the thesis provides preclinical evidence in support of the use of orexin receptor antagonists for the treatment of fear and anxiety associated with exposure to a stressful event. This would represent the first pharmacological treatment directed at an underlying cause of the condition.

It is well-known that orexin neurons innervate many brain areas that are involved in regulating stress, arousal and anxiety. One of the sites where orexin receptor antagonism could exert anxiolytic effects is the paraventricular nucleus of the thalamus

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(PVT). The PVT receives one of the most dense orexinergic projections in the brain (Kirouac, Parsons et al. 2005) and neurons of this midline thalamic area innervate regions of the prefrontal cortex and basal forebrain which are involved in the regulation of emotional behavior (Parsons, Li et al. 2007; Li and Kirouac 2008). Microinjections of orexin peptides into the PVT region have been shown to produce anxiety-like behaviors (Li, Li et al. 2009; Li, Li et al. 2010; Li, Li et al. 2010; Heydendael, Sharma et al. 2011) and microinjection of an $OX_2$ receptor antagonist in the paraventricular nucleus of the thalamus decreased the footshock-induce anxiety in the elevated-plus maze test (Li, Li et al. 2010). Another potential target for the anxiolytic effects of orexin antagonists is the bed nucleus of the stria terminalis where local injections of orexin-A was reported to produce anxiety in rats (Lungwitz, Molosh et al. 2012).

There are currently several companies including Acetelion GlaxoSmithKline, Hoffmann-La Roche, Merck, Sanofi, Biovitrum and Concert Pharmaceuticals which are developing orexin receptor antagonists. In fact, several dual orexin receptor antagonists have been developed and their effects on treating insomnia have been documented in clinical trials (Coleman and Renger 2010). It has been shown that the dual orexin receptor antagonists almorexant and suvorexant consistently and dose-dependently improved sleep duration and quality with limited side effect (Herring, Snyder et al. 2012; Hoever, de Haas et al. 2012; Sun, Kennedy et al. 2013). The results of experiments presented in this thesis support the potential usefulness of dual orexin receptor antagonists for the treatment of the fear and anxiety associated with PTSD.

As discussed earlier, orexins produce their effects by acting on $OX_1$ and $OX_2$ receptors. Those receptors distribute widely through the central nervous system. Studies

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have shown that $OX_1$ receptor is heavily expressed in the neurons located in the hippocampus, ventromedial hypothalamic nucleus, LC, raphe nuclei, whereas $OX_2$ receptor is predominantly expressed in the cerebral cortex, nucleus accumbens, midline thalamic nuclei, raphe nuclei, and many hypothalamic nuclei (Trivedi, Yu et al. 1998; Marcus, Aschkenasi et al. 2001). It was reported that microinjection of $OX_2$ receptor antagonist in to the midline thalamus attenuates the footshock-induced anxiety which cannot be blocked by $OX_1$ receptor antagonist (Li, Li et al. 2010). This indicates that the $OX_2$ receptor the midline thalamus play an important role in mediating anxiety.

## 4.9. Future Direction

Future studies should evaluate the contribution of the orexin receptor subtypes to the anxiolytic effect of the dual orexin receptor antagonists. The experiments may involve detecting the levels of $OX_1$ and $OX_2$ receptor in different brain areas, examining the pharmacological effect of specific orexin receptor antagonist, and testing the effect of local injection of orexin antagonist on the behavioral changes in the footshock model of PTSD. For example, it is possible that an $OX_2$ receptor antagonist may carry an advantage over a dual orexin receptor antagonist. To this date, there has been no clinical evaluation of potential use of orexin antagonist in the treatment of anxiety or PTSD. The availability of new dual orexin receptor and selective $OX_1$ and $OX_2$ receptor antagonists

with a limited side effect profile should provide opportunities for clinical researchers to investigate their potential benefits for treating patients with anxiety disorders and PTSD.

Clinical studies show that insomnia is highly prevalent in patients with PTSD (Belleville, Guay et al. 2009). It has also been shown that neurons in the PVT that receive innervation from orexin neurons send their projections to the forebrain which is involved in regulating sleep (Akert, Koella et al. 1952; Li and Kirouac 2008). This suggests that the orexin-PVT system may play an essential role in regulating sleep in people with PTSD. It is possible that an increase in the release of orexins in the PVT activates PVT neuron which leads to insomnia in PTSD. In order to test the hypothesis, I could examine whether the release of orexins in the PVT is increased in rats exposed to an intense stressor like footshocks. I could also examine the effect of blocking orexin receptors on PVT neuron with local injections of orexin antagonists on the activity of PVT neurons and sleep in animal models of PTSD. It would also be important to investigate whether it is possible to prevent of insomnia by inhibiting the activity of the orexin-PVT system.

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