The underlying mechanisms of the orexin (hypocretin) system in anxiety and fear

By

Huiying Wang

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Abstract

Anxiety and fear are emotions that are vital for individuals when dealing with difficult challenges and threats. However, persistent vigilance and frequent recall of memories associated with a fearful experience can lead to generalized anxiety disorder, posttraumatic stress disorder (PTSD), and phobias. Orexins (hypocretins) are neuropeptides that play a critical role in promoting arousal. Accumulating evidence suggests that orexin neurons are activated in animals exposed to stressful conditions and that orexins are involved in the regulation of anxiety and fear. In this thesis, the role of the orexins in anxiety and fear was studied using behavioral, biochemical, and pharmacological methods. Experiments were done to identify some of the possible mechanisms involved. First, microinjections of orexin peptides (orexin-A and orexin-B) into the paraventricular nucleus of the thalamus (PVT) induced anxiety-like behaviors. The anxiogenic effect produced by microinjections of orexin-A in the PVT was attenuated by blocking receptors for the stress-related peptides, corticotropin-releasing factor (CRF) and dynorphin. Second, the mRNA levels for prepro-orexin (ppOX) and the orexin 1 receptor (OX1R) were found to be increased in the posterior hypothalamus of shocked rats at 2 weeks post-shock. Third, 2 weeks after the footshocks, an enhanced level of CRF mRNA in the amygdala and ppOX mRNA in the posterior hypothalamus were detected in a subset of shocked rats that displayed an acute fear response to a novel tone and that

eventually resulted in a generalized anxiety (high responders, HR). Fourth, the dual orexin antagonist TCS1102 (20 mg/kg; i.p.) and OX1R antagonist SB334867 (20 and 30 mg/kg; i.p.) decreased fear level when tested at 2 weeks post-shock. These results suggest that endogenously released orexins regulate anxiety through acting on the PVT and that the brain kappa and CRF systems could be involved. The results also indicate that changes in orexins and CRF occur specifically in HR. These findings provide a better understanding of the brain mechanisms contributing to hyperarousal and anxiety. In addition, the results support the use of orexin receptor antagonists, especially OX1R antagonists, in the treatment of anxiety disorders including PTSD, panic attacks, and phobias.

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Dedication

I dedicate this thesis to my father, Yuxi Wang and my mother, Ling Wang for their unconditional love and support.

谨以此论文献给我的父亲王玉玺与我的母亲王玲，

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Table of Contents

List of Tables

List of Figures

Abbreviations

ACTH	adrenocorticotropic hormone
ANOVA	one-way analysis of variance
ASD	acute stress disorder
BST	bed nucleus of the stria terminalis
CeA	central nucleus of the amygdala
CRF	corticotropin-releasing factor
DMSO	dimethyl sulfoxide
DSM-III	diagnostic and statistical manual of mental disorders-third edition
FDA	food and drug administration
GABA	*gamma*-aminobutyric acid
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
hCRF	α-helical CRF
HPA	hypothalamic-pituitary-adrenal
HR	high responder
Hprt1	hypoxanthine phosphoribosyltransferase 1
i.c.v.	intracerebroventricular
i.p.	intraperitoneal
LR	low responder

NAc	nucleus accumbens
nBNI	norbinaltorphimine
OX1R	orexin-1 receptor
OX2R	orexin-2 receptor
PBS	phosphate buffered saline
ppOX	prepro-orexin
proENK	proenkephalin
PTSD	posttraumatic stress disorder
PVT	paraventricular nucleus of the thalamus
RT-PCR	reverse transcription-polymerase chain reaction
SAP	stretch-attend posture
SSRIs	selective serotonin reuptake inhibitors

Chapter 1
Introduction

1.1. Anxiety, Fear and Posttraumatic Stress Disorder (PTSD)

1.1.1. Anxiety and Fear

Anxiety and fear are emotional states elicited by an actual or potential threat to the survival of humans and animals (Steimer 2002, Tovote, Fadok, and Luthi 2015). These emotions can be characterized by a specific pattern of physiological, autonomic, hormonal and behavioral reactions (Anderson and Adolphs 2014, LeDoux 2000, 2014, Tovote, Fadok, and Luthi 2015). These reactions enable humans and animals to cope with a potential dangerous situation, avoid or reduce harm and ensure their survival. However, excessive and pathological anxiety and fear can interfere with the ability to cope with adverse or unexpected challenges and affect the hormone levels and normal behaviors of an organism (Steimer 2002, Tovote, Fadok, and Luthi 2015).

Although there are some similarities between anxiety and fear, certain key aspects of these two emotions are different (Davis et al. 2010). Fear is a strong emotion produced by a known threat. It can be triggered by the memory of a painful or stressful situation and it dissipates rapidly if the threat is removed (Davis et al. 2010, Lang, Davis, and Ohman 2000, Steimer 2002, Tovote, Fadok, and Luthi 2015). In contrast, anxiety is aroused by an unknown threat or internal conflict and it is a more long-lasting state (Davis et al. 2010, Lang, Davis, and Ohman 2000, Steimer

2002, Tovote, Fadok, and Luthi 2015). Fear elicited by a specific and real threat gives rise to active defensive response whereas anxiety elicited by a uncertain and potential threat leads to higher levels of arousal and vigilance (Davis et al. 2010, Steimer 2002). These differences provide a good perspective for studying the mechanisms of fear and anxiety in the laboratory.

1.1.2. General Introduction of PTSD

As a distinct anxiety disorder, posttraumatic stress disorder (PTSD) was initially introduced in the Diagnostic and Statistical Manual of Mental Disorders, Third Edition (DSM-III) in 1980 (American Psychiatric Association 1980). This anxiety disorder is characterized by evidence of an exposure to a traumatic event and the development of a combination of symptom clusters including re-experiencing, avoidance, negative emotions and cognition, and hyperarousal (American Psychiatric Association 2013, Levin, Kleinman, and Adler 2014, Weston 2014). Since then, this psychiatric disorder has attracted more and more attention for its relatively high prevalence and its considerable interpersonal, physical health, and societal consequences (Keane, Marshall, and Taft 2006, Warner et al. 2013). In the National Comorbidity Survey of U.S. population, Kessler and colleagues found that 60.7% of men and 51.2% of women were exposed to trauma at least once and that the estimated lifetime prevalence of PTSD in the United States was 7.8% (Kessler et al. 1995). In the National Comorbidity Survey Replication in 2005, the overall lifetime PTSD prevalence was found to be 6.8% and the rate of PTSD was the third highest of all the

anxiety disorders only falling behind specific phobia and social phobia (Kessler et al. 2005). The PTSD prevalence rates vary slightly in different countries and populations (Keane, Marshall, and Taft 2006). In Canada, a lifetime PTSD prevalence rate of 9.2% was estimated and 76.1% of respondents reported having been exposed to at least one traumatic event (Van Ameringen et al. 2008). It has been shown that the prevalence of PTSD is affected by a number of key factors, including sex, age, race, education level, marital status, region, and trauma type (Keane, Marshall, and Taft 2006, Kessler et al. 2005, Kessler et al. 1995, Warner et al. 2013).

PTSD is produced by directly or indirectly experiencing one or more traumatic events such as serious injury from accidents, natural disasters, sexual violence, combat warfare or any situation that causes an intense and lasting feeling of fear (Levin, Kleinman, and Adler 2014, Warner et al. 2013). Some individuals will subsequently develop symptom clusters: re-experiencing of the event in the forms of distressing memories, nightmares or flashbacks; avoidance of people, places or even distressing memories, thoughts that could serve as reminders of the traumatic event; negative changes in mood and cognition associated with the trauma; and hyperarousal experienced as hypervigilance, anxiety, nervousness and sleep disturbance (American Psychiatric Association 2013, Levin, Kleinman, and Adler 2014, Warner et al. 2013). If the acute response mentioned above occurs in 2 days to 1 month after the traumatic event, it is defined as acute stress disorder (ASD) (Cardena and Carlson 2011). When these symptoms persist more than 1 month, the diagnosis of PTSD is given

(American Psychiatric Association 2013). If the diagnosis is made 6 months after the traumatic event, it's called delayed onset PTSD (American Psychiatric Association 2013, Levin, Kleinman, and Adler 2014, Warner et al. 2013).

PTSD causes significant distress and interferes with daily living activities (Keane, Marshall, and Taft 2006, Warner et al. 2013). For example, patients with PTSD have poorer physical health and life satisfaction and they are more likely to have problems in their family life and social activities (Keane, Marshall, and Taft 2006). In addition, PTSD is strongly comorbid with other mental disorders, such as major depressive disorder, social phobia, anxiety disorders and substance abuse (Kessler et al. 1995, Perkonigg et al. 2000, Van Ameringen et al. 2008). Patients with PTSD also have high suicidal tendencies as one in five of the patients attempt suicide (Davidson et al. 1991, Warner et al. 2013). It is vital to study the underlying neurobiological basis for PTSD which could lead to the development of new and more effective treatments.

1.2. Orexins System

Recently, a family of hypothalamic neuropeptides called orexins (hypocretins) have been studied for its role in the regulation of fear and anxiety (Flores et al. 2014, Furlong et al. 2009, Sears et al. 2013, Soya et al. 2013, Steiner, Lecourt, and Jenck 2012). The orexin peptides have been shown to play a key role in maintaining behavioral arousal states in animals including wakefulness, exploration of novel environment, food seeking and intake, copulation, and many other behaviors associated with survival (Sakurai 2007, 2014). Anxiety disorder including PTSD

involve a significant degree of hyperarousal and a number of studies show the orexins contribute to the hyperarousal states associated with these disorders (Levin, Kleinman, and Adler 2014, Warner et al. 2013, Flores et al. 2014, Furlong et al. 2009, Sears et al. 2013, Soya et al. 2013, Steiner, Lecourt, and Jenck 2012). The aim of the experiments presented in this thesis was to examine how the orexin system contribute to fear and anxiety associated with an acute and relatively intense fear experience.

1.2.1. Orexins and Orexin Receptors

Since discovered in 1998, the orexins have attracted a lot of attention (de Lecea et al. 1998, Sakurai et al. 1998). Due to their role in food consumption, the neuropeptides were named orexins after the Greek word *orexis* which means appetite (Sakurai et al. 1998). Another group discovered the neuropeptides simultaneously and named them hypocretins because they shared some sequence identity with members of the metabolic hormones called incretins and because the orexin peptides were found in the hypothalamus (de Lecea et al. 1998). The most intriguing part of this discovery is that these orexin neurons are exclusively distributed in the lateral hypothalamus but their fibers have widespread distribution in the whole brain (de Lecea et al. 1998, Sakurai et al. 1998). The extensive projections of the orexin system suggest their role in a wide range of behaviors, such as feeding, arousal, emotion and addiction (Boutrel, Cannella, and de Lecea 2010, Giardino and de Lecea 2014, Li, Hu, and de Lecea 2014, Sakurai 2002, 2014, Sakurai and Mieda 2011, Tsujino and Sakurai 2009, 2013).

The orexin peptides are found to have two bioactive forms called orexin-A and orexin-B (also known as hypocretin 1 and hypocretin 2) (de Lecea et al. 1998, Sakurai et al. 1998). They are produced from cleaving the same precursor peptide, prepro-orexin (ppOX) which is a 130-residue polypeptide (rodent) (de Lecea et al. 1998, Sakurai et al. 1998). Orexin-A is composed of 33-amino acid (3,562 Da) with two sets of intrachain disulfide bonds while orexin-B is composed of 28-amino acid (2,937 Da) (de Lecea et al. 1998, Sakurai et al. 1998). Because of having two intrachain disulfide bonds, orexin-A is more stable than orexin-B which is a linear peptide (Kastin and Akerstrom 1999, Spinazzi et al. 2006). The two orexin peptides show 46% (13/28) amino acid identity (Matsuki and Sakurai 2008). The C-terminal half of orexins is very similar (73%; 11/15) while the N-terminal half shows significant differences in amino acid sequences (Matsuki and Sakurai 2008). Orexin sequences have been identified from mammals (human, mouse, rat, pig, dog), amphibians (Xenopus), birds (chicken) to teleosts (goldfish) and the genetic and molecular structures of orexins are relatively well conserved across vertebrate species (Alvarez and Sutcliffe 2002, Shibahara et al. 1999, Wong et al. 2011). The sequences of orexins are highly conserved especially among mammalian species (Sakurai et al. 1998, Wong et al. 2011). Orexin-A is identical across mammalian species and orexin-B only has 1 or 2 amino acid substitutions from human to pig and rat (Sakurai et al. 1998, Wong et al. 2011). In addition, ppOX sequences in rat are 83% and 95% identical to their counterparts in human and mouse (Sakurai et al. 1998).

This suggests a strong evolutionary pressure to preserve structure and the physiological functions of orexins.

The orexins act at two G protein-coupled receptors (GPCR) called the orexin-1 receptor (OX1R) and the orexin-2 receptor (OX2R) (Sakurai et al. 1998). There is 64% identity between the deduced full-length human OX1R and OX2R (Sakurai et al. 1998). Similar to the orexin peptides, both of the orexin receptors are highly conserved across mammalian species (Sakurai et al. 1998, Wong et al. 2011). For example, the identities of orexin receptors between human and rat are 94% for OX1R and 95% for OX2R (Sakurai et al. 1998). However, only OX2R has been identified in non-mammalian species (Wong et al. 2011). It suggests that OX2R may be evolutionary more ancient and OX1R is specific to mammals. Moreover, the binding affinities of orexins for the two receptors are different. Studies demonstrate that the OX1R has a greater affinity for orexin-A than for orexin-B whereas the OX2R has similar affinity for both orexin-A and orexin-B, suggesting that the OX1R is selective for orexin-A while the OX2R is a nonselective receptor (Matsuki and Sakurai 2008, Sakurai et al. 1998).

Orexins depolarize neurons and increase the cell excitability and firing rate through presynaptic and postsynaptic modulation (Arrigoni, Mochizuki, and Scammell 2010, Hagan et al. 1999, Liu, van den Pol, and Aghajanian 2002, Scammell and Winrow 2011). The signal transduction pathways coupled to orexin receptors have been extensively studied in cells transfected with OX1R or OX2R, however, the contribution of G proteins and subsequent signaling mechanisms remain unclear

(Scammell and Winrow 2011, Zhang et al. 2013, Zhu et al. 2003). Both of the orexin receptors have been identified to couple to G_q proteins and the OX2R is also shown to couple to G_i/G_o proteins which may result in inhibition effects (Scammell and Winrow 2011, Zhang et al. 2013, Zhu et al. 2003). The effects of orexins on target neurons are mediated by modulating several ion channels (Hoang et al. 2003, Scammell and Winrow 2011, Zhang et al. 2013). For example, some neurons may get activated by the inhibition of potassium channels, such as G protein-regulated inward rectifier channels (Hoang et al. 2003). In addition, the intracellular calcium concentration is elevated after orexins binding to the orexin receptors by intracellular calcium release or extracellular calcium influx through activation of the voltage-gated calcium channels and nonselective cation channels (Scammell and Winrow 2011, Zhang et al. 2013). The calcium influx could activate the sodium/calcium exchanger and lead to excitation of neurons (Scammell and Winrow 2011, Zhang et al. 2013). Moreover, orexin signaling may increase the number of other receptors in the cell membrane including the N-methyl-D-aspartate (NMDA) receptors and induce higher responsiveness to the excitation of glutamate (Borgland, Storm, and Bonci 2008, Scammell and Winrow 2011).

1.2.2. Distributions of Orexin Neurons and Orexin Receptors

Orexin neurons are exclusively located in the lateral regions of the hypothalamus, including the perifornical area, lateral and posterior hypothalamic area (Matsuki and Sakurai 2008, Sakurai et al. 1998). It has been estimated that the number of orexin

neurons is around 3,000 in rat brain and around 70,000 in human brain (Sakurai and Mieda 2011). This relatively small population of orexin neurons send widespread projections throughout the entire brain including the ventral tegmental area, paraventricular nucleus of the thalamus (PVT), locus coeruleus, dorsal raphe nucleus, and amygdala (Mondal, Nakazato, and Matsukura 2000, Nambu et al. 1999). Orexin receptors are widely distributed in regions which receive orexin projections. The OX1R and OX2R mRNAs are expressed in partially overlapping areas which suggest that the orexin receptors may play different roles in physiological functions (Johnson, Molosh, et al. 2012, Marcus et al. 2001). Both OX1R and OX2R are expressed in several brain regions, including PVT, ventral tegmental area and hypothalamus (Johnson, Molosh, et al. 2012, Marcus et al. 2001). In addition, some brain regions mainly express one of the orexin receptors (Johnson, Molosh, et al. 2012, Marcus et al. 2001). For example, the bed nucleus of the stria terminalis (BST) and the cingulate cortex mainly express OX1R while the nucleus accumbens (NAc) and the tuberomammillary nucleus mainly express OX2R (Johnson, Molosh, et al. 2012, Marcus et al. 2001).

It has been shown that the orexin receptors are highly expressed in brain regions associated with the regulation of arousal and emotion (Mahler et al. 2012, Sakurai 2014, Sakurai et al. 1998). Brain areas which produce monoamine transmitters involved in arousal are densely innervated by orexin fibers and that these monoamine transmitters include norepinephrine, serotonin, histamine, and dopamine (Alexandre, Andermann, and Scammell 2013, Cutler et al. 1999, Date et al. 1999, Nambu et al.

1999, Peyron et al. 1998). The neurons producing these monoamine transmitters contain a large number of orexin receptors, including the norepinephrine neurons in the locus coeruleus (Carter et al. 2012, Hagan et al. 1999), the serotonin neurons in the dorsal raphe (Tao et al. 2006), the histamine neurons in the tuberomammillary nucleus (Takahashi, Lin, and Sakai 2006, Thakkar 2011), and the dopamine neurons in the ventral tegmental area (Vittoz and Berridge 2006).

1.2.3. General Functions of Orexins

The widespread projections of orexin neurons suggest multiple functions of orexins. Orexin neurons are found in the lateral hypothalamus which is recognized as a feeding center indicating that orexins play a key role in feeding behavior as well as energy expenditure (Sakurai 2014, Sakurai et al. 1998). Moreover, orexin neurons project to some brain areas related to reward and addiction, including the ventral tegmental area, PVT and locus coeruleus (Mahler et al. 2012). Orexin neurons also send projections to the premotor autonomic centers, such as the periaqueductal gray, parabrachial nucleus, nucleus of the solitary tract, and paraventricular nucleus of the hypothalamus, suggesting that the orexin system is also involved in regulating autonomic functions (Kuwaki and Zhang 2012, Sakurai 2014). In addition, orexin neurons project to many of the nuclei which regulate arousal including the PVT, locus coeruleus, dorsal raphe nucleus, and tuberomammillary nucleus (Cutler et al. 1999, Date et al. 1999, Nambu et al. 1999, Peyron et al. 1998). The dense orexin projections to other brain arousal centers indicate that one of the key

functions of orexins is to maintain behavioral arousal (Berridge, Espana, and Vittoz 2010, Boutrel, Cannella, and de Lecea 2010, Sakurai 2007, Taheri, Zeitzer, and Mignot 2002).

1.2.4. The Role of Orexins in Arousal

Compelling evidence has demonstrated that orexins are arousal-promoting peptides which play an important role in maintaining wakefulness (Sakurai 2002, Sutcliffe and de Lecea 2002). Knockout of the ppOX gene in mice or mutation of OX2R gene in dogs leads to a difficulty in maintaining wakefulness (Chemelli et al. 1999, Lin et al. 1999). In fact, a loss of orexin signaling appears to be the primary cause of a sleep disorder called narcolepsy in which an individual can literally fall asleep instantly during the daytime (Sakurai 2007, Taheri, Zeitzer, and Mignot 2002). Clinical studies showed that human narcolepsy patients had an 85% - 95% loss of orexin neurons and significant low levels of orexin-A in cerebrospinal fluid (Mignot et al. 2002, Peyron et al. 2000, Thannickal et al. 2000). Central administration of orexin-A in rats increased the baseline level of wakefulness in both sleep and wake periods (Espana, Plahn, and Berridge 2002). Moreover, oral administration of a dual orexin receptor antagonist significantly reduced the level of wakefulness in rat, dog and human (Brisbare-Roch et al. 2007). Thus, several orexin antagonists have been developed for the treatment of insomnia and suvorexant, one of the dual orexin receptor antagonist, has been approved to be used for the treatment of insomnia in U.S. and Japan in 2014 (Sutton 2015, Yeoh et al. 2014).

In addition, a large body of evidence demonstrates that the activity of orexin neurons fluctuates with arousal level (Espana, Valentino, and Berridge 2003, Estabrooke et al. 2001, Mileykovskiy, Kiyashchenko, and Siegel 2005, Saper, Scammell, and Lu 2005, Yoshida et al. 2001). It has been shown that orexin neurons have maximal level of activity during active wakefulness, such as exploration and foraging (Lee, Hassani, and Jones 2005, Mileykovskiy, Kiyashchenko, and Siegel 2005). Furthermore, orexin neurons are found to decrease their discharge rate in low arousal conditions and that they nearly cease firing during sleep (Lee, Hassani, and Jones 2005, Mileykovskiy, Kiyashchenko, and Siegel 2005). These findings indicate that the orexin neurons respond differently to distinct arousal conditions and that the orexins could be one of the main modulators of behavioral arousal.

As mentioned above, orexin neurons innervate brain regions that regulate arousal levels and they also innervate brain regions which regulate motivation and emotion (Mahler et al. 2012, Sakurai 2014, Sakurai et al. 1998). Orexin neurons also send projections to brain regions which regulate autonomic and motor functions (Hu et al. 2015, Kuwaki and Zhang 2012, Sakurai 2014). In addition, it has been shown that administrations of orexins in the lateral ventricle produced c-Fos immunoreactivity in a number of brain areas, such as locus coeruleus, dorsal raphe nucleus, PVT, paraventricular nucleus of the hypothalamus, and nucleus of the solitary tract, and that these areas mediate the behavioral responses to challenging situations (Alexandre, Andermann, and Scammell 2013). Consequently, the efferent projections of orexin neurons suggest that the release of orexins may affect the activity of neurons in these

brain regions that regulate arousal level as well as functions related to the survival of organisms.

It is well documented that the monoamine transmitters norepinephrine, serotonin, histamine, and dopamine are involved in the modulation of arousal (Aston-Jones and Bloom 1981, Berridge and Waterhouse 2003, Takahashi, Lin, and Sakai 2006, Tao et al. 2006, Thakkar 2011, Vittoz and Berridge 2006). Recent evidence indicates that orexins may modulate the activity of neurons that produce arousal-promoting monoamine transmitters and enhance the release of these neurotransmitters (Alexandre, Andermann, and Scammell 2013, Carter et al. 2012, Hagan et al. 1999, Tao et al. 2006, Vittoz and Berridge 2006). For example, the application of orexin-A elevated the cell firing of norepinephrine neurons in the locus coeruleus which play a role in promoting arousal (Aston-Jones and Bloom 1981, Berridge and Waterhouse 2003, Hagan et al. 1999). Orexin-A was also found to excite the histamine neurons in the tuberomammillary nucleus through OX2R resulting in an increase of arousal level (Alexandre, Andermann, and Scammell 2013). In addition, when infused in the dorsal raphe nucleus or pontine reticular formation, orexin-A increased the release of serotonin (Tao et al. 2006). Orexins innervate dopamine neurons and modulate the activity of these neurons and the release of dopamine (Korotkova et al. 2003, Moorman and Aston-Jones 2010, Narita et al. 2006, Vittoz and Berridge 2006). For example, infusion of orexin-A in the ventral tegmental area or lateral ventricle could increase the dopamine level in the prefrontal cortex (Vittoz and Berridge 2006). These studies point to the critical role of orexins in the maintaining of behavioral

arousal. In summary, orexins might orchestrate the activity of these downstream effectors and enhance the release of most arousal-promoting neurotransmitters which in turn coordinate the arousal state of organisms (Alexandre, Andermann, and Scammell 2013, Kumar, Chanana, and Choudhary 2016).

1.2.5. The Role of Orexins in Stress

It is now well established that orexins are involved in the regulation of the stress response (Johnson, Molosh, et al. 2012, Tsujino and Sakurai 2013). The orexin system is found to be activated by many stressors, such as immobilization, cold exposure, conditioned fear, footshocks and forced swim (Chang et al. 2007, Furlong et al. 2009, Ida et al. 2000, Sakamoto, Yamada, and Ueta 2004, Winsky-Sommerer et al. 2004). The level of ppOX mRNA was increased by restraint which suggests that restraint stress increases the synthesis of orexins (Reyes et al. 2003). In addition, orexins play a role in some stress-induced response, including the activation of hypothalamic-pituitary-adrenal (HPA) axis and the release of adrenocorticotropic hormone (ACTH) and corticosterone (Winsky-Sommerer, Boutrel, and de Lecea 2005). Orexin knockout mice showed diminished physiological response in the resident-intruder test (Kayaba et al. 2003). Anatomical evidence shows that orexin receptors are expressed in every aspect of the HPA axis in the brain indicating that orexins are an important regulator of HPA (Date et al. 2000, Lopez et al. 1999, Spinazzi et al. 2006, Trivedi et al. 1998).

Furthermore, activation of the orexin system induces stress-like effects (Tsujino and Sakurai 2013). Infusion of orexin-A into the cerebral ventricle increases arterial blood pressure, heart rate, cerebral blood flow (Dun et al. 2000, Shirasaka et al. 1999) and body temperature (Yoshimichi et al. 2001). Moreover, the activation of HPA axis by orexins stimulates the release of ACTH and corticosterone (Al-Barazanji et al. 2001, Jaszberenyi et al. 2000, Kuru et al. 2000, Samson et al. 2002) and induces stress-like behaviors, such as grooming, face washing, locomotor activity, chewing of inedible material and increased food consumption (Berridge, Espana, and Vittoz 2010, Dube, Kalra, and Kalra 1999, Espana, Plahn, and Berridge 2002, Haynes et al. 1999, Ida et al. 2000, Li, Hu, and de Lecea 2014).

It has been shown that a corticotropin-releasing factor (CRF)-mediated mechanism is involved in the activation of the orexin system in stressful conditions (Sakurai 2014, Winsky-Sommerer, Boutrel, and de Lecea 2005, Winsky-Sommerer et al. 2004). Anatomical evidence has shown the reciprocal connections between orexin neurons in the lateral hypothalamus and CRF neurons in the paraventricular nucleus of the hypothalamus (Marcus et al. 2001, Winsky-Sommerer et al. 2004). Terminals of CRF neurons make direct synaptic contacts with orexin neurons in the lateral hypothalamus and numerous CRF receptors are expressed by the orexin neurons suggesting that CRF could modulate the activity of orexin neurons (Winsky-Sommerer et al. 2004). Correspondingly, orexin neurons send dense projections to the paraventricular nucleus of the hypothalamus and orexin receptors are abundantly expressed by the CRF neurons in the nucleus (Marcus et al. 2001). A

number of findings are in accordance with this anatomical evidence. CRF depolarized the membrane potential of hypothalamic slices containing orexin neurons and increased the firing rate of orexin neurons (Winsky-Sommerer et al. 2004). CRF receptor 1 knockout mice showed significantly lower level of activation of orexin neurons in response to footshock and restraint stress (Winsky-Sommerer et al. 2004). In addition, central administration of α-helical CRF (hCRF), which is a nonselective CRF receptor antagonist, blocked orexin induced stress-like behaviors such as grooming and face washing (Ida et al. 2000). The increase in corticosterone release induced by the infusion of orexins was also blocked by hCRF (Jaszberenyi et al. 2000, Samson et al. 2002). On the other hand, a single injection of orexin-A in the cerebral ventricle elevated the number of activated CRF neurons in the paraventricular nucleus of the hypothalamus to up to approximately 96% and increased the CRF mRNA level in the same brain region (Al-Barazanji et al. 2001, Sakamoto, Yamada, and Ueta 2004). Pretreatment with an OX1R antagonist before the stress exposure blocked the upregulation of CRF mRNA in the paraventricular nucleus of the hypothalamus (Heydendael et al. 2011). Taken together, CRF could be a candidate for stimulating the release of orexins which in turn contributes to the high level of arousal in response to stressful situations.

1.2.6. The Role of Orexins in Fear and Anxiety

As mentioned above, there are differences between anxiety and fear which provides a good perspective for the use of animal models in studying the mechanisms

of fear and anxiety in the laboratory. In rodents, fear is modeled by pairing of an aversive unconditioned stimulus, such as footshock, with a neutral conditioned stimulus, such as a context or a cue (LeDoux 2000). This paradigm is called fear conditioning and according to the type of conditioned stimulus used (context or sensory cues like an auditory tone), conditioning paradigms are called contextual fear conditioning and cued fear conditioning (Curzon, Rustay, and Browman 2009, Rozeske et al. 2015). Contextual fear conditioning involves placing a rodent in a novel environment and providing an aversive event like footshock while cued fear conditioning adds a neutral cue like tone or light to the context (Curzon, Rustay, and Browman 2009, Rozeske et al. 2015). A freezing response, which is a species-specific response to fear, is usually analyzed to show the fear level of rodents (Blanchard and Blanchard 1988). Anxiety is modeled by exposing rodents to either aversive situations, such as open spaces, or situations related to aversive stimulus in which the rodents show avoidance behavior (Steimer 2002, 2011). The tests of anxiety are usually based on the conflict between the avoidance of aversive situations and approach of preferred situations as exemplified by the elevated plus maze, elevated T-maze, open field, social interaction and dark/light box (Steimer 2002, 2011).

A potential role for orexins in fear and anxiety is supported by the anatomical evidence that bidirectional projections exist between orexin neurons and brain regions associated with motivation and emotion (Johnson, Molosh, et al. 2012, Mondal, Nakazato, and Matsukura 2000, Nambu et al. 1999, Sakurai et al. 2005, Yoshida et al.

2006). In particular, orexin terminals are found densely in the prefrontal cortex, BST, locus coeruleus, PVT and amygdala (Johnson, Molosh, et al. 2012, Mondal, Nakazato, and Matsukura 2000, Nambu et al. 1999). Orexin neurons also receive abundant projections from these same brain regions (Sakurai et al. 2005, Yoshida et al. 2006).

Pharmacological evidence indicates that the baseline synthesis or release of orexins may contribute to fear (Furlong et al. 2009, Soya et al. 2013, Steiner, Lecourt, and Jenck 2012). A dual orexin receptor antagonist called almorexant attenuated fear-induced startle (Steiner, Lecourt, and Jenck 2012) and this drug reduced the high levels of heart rate and blood pressure which were induced by the chamber where the rats were previously exposed to footshocks (Furlong et al. 2009). Furthermore, evidence from knockout mice supports the role of OX1R in cued and contextual fear conditioning paradigms and the role of OX2R in contextual fear conditioning (Soya et al. 2013).

Orexins have also been found to be involved in forming (learning) and forgetting (extinction) of fear memories (Flores et al. 2014, Sears et al. 2013). Rats that received a selective OX1R antagonist called SB334867 showed a significantly lower level of fear (Sears et al. 2013). This drug has also been reported to reduce fear in contextual and cued fear conditioning paradigms and genetic deletion of OX1R in mice showed similar effects (Flores et al. 2014). An OX2R antagonist called TCSOX229 reduced fear in a contextual fear conditioning paradigm while it had no effect in cued fear conditioning (Flores et al. 2014). Moreover, administration of

orexin-A significantly impaired the extinction process in both contextual and cued fear conditioning tests (Flores et al. 2014). Pharmacological blockade of OX1R with SB334867 facilitated the extinction of fear in contextual and cued fear conditioning paradigms (Flores et al. 2014). In contrast, administration of orexin-B or blockade of OX2R with TCSOX229 did not have effects on extinction process of contextual and cued fear (Flores et al. 2014).

Interestingly, a recent study examined the effect of the orexin receptor antagonist almorexant on fear generalization after a single footshock exposure (Viviani et al. 2015). They tested the generalized avoidance in situations with high similarity and low similarity to the context where the rats received footshocks and found that almorexant significantly decreased the fear generalization in the low similarity situation and had no effects in the high similarity situation (Viviani et al. 2015). The results suggested that the orexin antagonist specifically reduced the fear generalization without impairing the acquired aversive memory of contextual fear.

A number of groups have shown that orexins could induce anxiety states in rodents by injections of orexins in brain areas associated with anxiety, including the midline thalamus (Heydendael et al. 2011, Li et al. 2009, Li et al. 2010), BST (Lungwitz et al. 2012) and lateral ventricles (Suzuki et al. 2005). Another group also reported that the anxiety displayed in a rat model of panic disorder was attenuated by systemic injections of the OX1R antagonist SB334867 or a dual orexin receptor antagonist called DORA-12 (Johnson et al. 2015, Johnson, Samuels, et al. 2012, Johnson et al. 2010). In addition, the knockout of OX1R in mice increased the

acoustic startle response and the anxiety in the elevated plus maze and social interaction test (Abbas et al. 2015).

In summary, the specific distribution of orexin neurons and fibers support the role of orexins in arousal and a wide variety of behaviors, including fear and anxiety which are typical symptoms of PTSD. While the pathway for orexin mediated fear and anxiety are not known, the brain areas which contain dense orexin fibers and are associated with arousal and emotion may be involved.

1.3. Paraventricular Nucleus of the Thalamus (PVT)

The midline and intralaminar thalamic nuclei located in the dorsal thalamus are hypothesized to be involved in important physiological mechanisms including arousal, attention and consciousness (Berendse and Groenewegen 1991, Groenewegen and Berendse 1994). The PVT is a distinct nucleus among these midline and intralaminar thalamic nuclei for its very dense innervations from orexin neurons of the hypothalamus (Kirouac, Parsons, and Li 2005). In fact, adjacent midline and intralaminar thalamic nuclei receive relatively weak to moderate orexin projections from orexin neurons (Kirouac, Parsons, and Li 2005). Studies using in vitro patch-clamp recordings show that most neurons in the PVT could be depolarized and can be induced to fire at a higher rate by the orexin-A and orexin-B peptides (Huang, Ghosh, and van den Pol 2006, Kolaj et al. 2007). Besides the orexin fibers, the PVT contains fibers containing a large number of peptides including dynorphin, enkephalin, substance P, cholecystokinin, CRF, neuropeptide S, neuropeptide Y,

melanin-concentrating hormone, and cocaine- and amphetamine-related peptide (Battaglia, Colacitti, and Bentivoglio 1992, Clark et al. 2011, Kirouac 2015, Kirouac, Parsons, and Li 2006, Lee, Lee, and Lee 2015, Otake and Nakamura 1995, Parsons, Li, and Kirouac 2006) and all of the monoamines including dopamine, norepinephrine, epinephrine, and serotonin (Kirouac 2015, Li, Shi, and Kirouac 2014, Otake and Ruggiero 1995).

1.3.1. Sources of Inputs to the PVT

It has been shown that the PVT receives inputs from neurons scattered throughout the hypothalamus (Chen and Su 1990, Cornwall and Phillipson 1988, Kirouac 2015, Kirouac, Parsons, and Li 2006, Li and Kirouac 2012, Otake and Ruggiero 1995). The dorsomedial nucleus, suprachiasmatic nucleus, arcuate nucleus, medial preoptic area and lateral hypothalamus project densely to the anterior and posterior PVT (Chen and Su 1990, Cornwall and Phillipson 1988, Kirouac 2015, Kirouac, Parsons, and Li 2006, Li and Kirouac 2012, Otake and Ruggiero 1995). The dorsomedial nucleus and suprachiasmatic nucleus, which provide the most robust projections to the PVT, are major modulators of circadian rhythms suggesting that the PVT may be involved in the regulation of behaviors associated with rhythms (Kirouac 2015, Saper, Scammell, and Lu 2005). In addition, the PVT receives innervation from a dense cluster of neurons in the reticular nucleus of the thalamus which plays a pivotal role in regulating gustatory and visceral sensory information and attention (Chen and Su 1990, Cornwall and Phillipson 1988, Guillery, Feig, and Lozsadi 1998,

Kirouac 2015, Kirouac, Parsons, and Li 2006, Li and Kirouac 2012, Otake and Ruggiero 1995, Pinault 2004, Stehberg et al. 2001).

Besides projections from the hypothalamus and thalamus, the PVT receives significant inputs from several nuclei in the brainstem including the locus coeruleus, parabrachial nucleus, periaqueductal gray, raphe nuclei, pedunculopontine tegmental nucleus and nucleus of the solitary tract (Chen and Su 1990, Cornwall and Phillipson 1988, Kirouac 2015, Kirouac, Parsons, and Li 2006, Li and Kirouac 2012). These nuclei in the brainstem are prominently involved in regulating visceral and nociceptive functions (Cechetto 1987, Heinricher et al. 2009, Kirouac 2015). For example, the parabrachial region is recognized as a relay of visceral and nociceptive signals and the periaqueductal gray plays a key role in the regulation of nociception (Cechetto 1987, Heinricher et al. 2009, Kirouac 2015).

In addition to innervation from the brainstem, both the anterior PVT and posterior PVT receive innervation from layer 6 of the medial prefrontal cortex including the prelimbic, infralimbic, and insular cortical areas (Li and Kirouac 2012). Meanwhile, the PVT is a major source of input to these areas of prefrontal cortex (Li and Kirouac 2012). However, there appears to be differences in the source of cortical inputs to the anterior PVT and posterior PVT (Chen and Su 1990, Li and Kirouac 2012). For example, the posterior PVT receives much denser projections from the insular and infralimbic cortices than the anterior PVT (Chen and Su 1990, Li and Kirouac 2012). The anterior PVT is innervated more robustly by the subiculum of the hippocampus than the posterior PVT (Chen and Su 1990, Li and Kirouac 2012).

Furthermore, it has been shown that the posterior insular cortex is involved in the gustatory and visceral sensory functions (Cechetto and Saper 1987, Kosar, Grill, and Norgren 1986, Shi and Cassell 1998) and the prelimbic and infralimbic areas are involved in executive functions (Kesner and Churchwell 2011). The subiculum is associated with the regulation of stress, fear, anxiety and motivation (McNaughton 2006, O'Mara et al. 2009). Therefore, the PVT may participate in the modulation of various functions and play as a relay center that integrate the input information and select appropriate responses.

1.3.2. Target Areas for the PVT

Anterograde tracing studies have shown that many brain areas are innervated by the PVT (Li and Kirouac 2008, Moga, Weis, and Moore 1995, Pinto, Jankowski, and Sesack 2003, Vertes and Hoover 2008). Among all of the target areas, the NAc as well as the extended amygdala which is an anatomical macro-structure including the BST, central nucleus of the amygdala (CeA) and interstitial nucleus of the posterior limb of the anterior commissure receives the most intense projections from the PVT (Kirouac 2015, Li and Kirouac 2008). Both the core and the shell of the NAc are heavily innervated by the PVT suggesting that the PVT modulates behaviors through the regulation of the NAc such as food intake and drug addiction (Li and Kirouac 2008, Moga, Weis, and Moore 1995, Pinto, Jankowski, and Sesack 2003, Vertes and Hoover 2008). The fiber terminals from PVT neurons make synaptic contact with the axons and dendrites of neurons in the NAc (Pinto, Jankowski, and Sesack 2003).

The NAc neurons produce enkephalin and dynorphin which are associated with emotional state (Furuta, Zhou, and Kaneko 2002, Kelley, Baldo, and Pratt 2005, Pennartz, Groenewegen, and Lopes da Silva 1994, Zhou, Furuta, and Kaneko 2003). In addition, the medial and ventral parts of the caudate putamen in the striatum which also produce opioid peptides like enkephalin and dynorphin (Fallon and Leslie 1986, Gago et al. 2013, Guttenberg et al. 1996) and these same regions also receive a dense projections from the PVT (Li and Kirouac 2008, Moga, Weis, and Moore 1995, Vertes and Hoover 2008).

The PVT sends a most significant projection to dorsolateral BST and lateral and capsular CeA, which are key components of the brain's fear and anxiety systems (Li and Kirouac 2008, Moga, Weis, and Moore 1995, Vertes and Hoover 2008). The fiber terminals from the PVT appear to make direct synaptic contacts with CRF neurons in the BST and CeA (Kirouac 2015, Li and Kirouac 2008). The heavy projections from the PVT have been shown to overlap with the areas in the NAc and extended amygdala that contain dense dopamine fibers that most likely originate from the ventral tegmental area (Freedman and Cassell 1994, Parsons, Li, and Kirouac 2007). Furthermore, the extended amygdala areas that are rich in the CRF neurons also contain neurons that express other neuropeptides including dynorphin, enkephalin, neurotensin and somatostatin (Cassell, Freedman, and Shi 1999, Marchant, Densmore, and Osborne 2007, Poulin et al. 2009). In addition, other regions of the amygdala are innervated by the PVT including the the basomedial,

basolateral, medial, and lateral nuclei of the amygdala (Li and Kirouac 2008, Moga, Weis, and Moore 1995, Vertes and Hoover 2008).

As mentioned earlier, some areas of the cerebral cortex also receive innervation from the PVT including the infralimbic cortex, prelimbic cortex and insular cortex (Li and Kirouac 2008, Moga, Weis, and Moore 1995, Pinto, Jankowski, and Sesack 2003, Vertes and Hoover 2008). Anterograde studies have reported that all layers of the infralimbic cortex and ventral prelimbic cortex receive fibers from the PVT and that the agranular and dysgranular portions of the insular cortex are also innervated by the PVT (Li and Kirouac 2008, Moga, Weis, and Moore 1995, Vertes and Hoover 2008). The role of the infralimbic cortex and ventral prelimbic cortex in visceromotor control, cognition, emotional control, goal-directed behaviors and executive function has been reported by many studies (Euston, Gruber, and McNaughton 2012, Heidbreder and Groenewegen 2003, Kesner and Churchwell 2011, Vertes 2006). Moreover, the insular cortex plays a role in processing multi-modal sensory inputs including visceral, gustatory, nociceptive and thermal information (Cechetto and Saper 1987, Kobayashi 2011, Kosar, Grill, and Norgren 1986, Shi and Cassell 1998). These projections place the PVT in a position to integrate a variety of inputs and modulate a wide range of behaviors associated with emotional and motivational behaviors.

In addition to the brain areas mentioned above, the PVT also projects to several areas in the hypothalamus and hippocampus (Kirouac 2015, Li and Kirouac 2008, Moga, Weis, and Moore 1995, Vertes and Hoover 2008). Many areas in the hypothalamus including the suprachiasmatic nucleus, arcuate nucleus, dorsomedial

nucleus, ventromedial nucleus, lateral hypothalamus and lateral septal area are innervated by the relatively less dense projections of the PVT (Kirouac 2015, Li and Kirouac 2008, Moga, Weis, and Moore 1995, Vertes and Hoover 2008). Furthermore, the subiculum of the hippocampus which is involved in regulating the processes of emotion and motivation receives projections from the anterior PVT (Li and Kirouac 2008, McNaughton 2006, O'Mara et al. 2009).

1.3.3. Functions of the PVT

The various afferents and efferents suggest the type of behavioral functions that the PVT may be involved with. The heavy projections to the NAc, prefrontal context and amygdala point to the critical role of the PVT in motivational behaviors, such as food reward and drug seeking (Li and Kirouac 2008, Moga, Weis, and Moore 1995, Pinto, Jankowski, and Sesack 2003, Vertes and Hoover 2008). Several lines of evidence show that the PVT plays a role in the regulation of food intake and food reward behaviors (Bhatnagar and Dallman 1999, Choi et al. 2010, Igelstrom, Herbison, and Hyland 2010, Stratford and Wirtshafter 2013). For instance, ibotenate lesions of the posterior PVT or inactivation of the PVT with injections of a *gamma*-aminobutyric acid (GABA) agonist muscimol increased food intake (Bhatnagar and Dallman 1999, Stratford and Wirtshafter 2013). Following exposure of cues or contexts associated with palatable food, the expression of c-Fos, which is a well-known marker of neuronal activation, was significantly increased in the PVT suggesting that the PVT neurons were activated in the food reward conditions (Choi

et al. 2010, Igelstrom, Herbison, and Hyland 2010). Furthermore, experimental evidence indicates a role for the PVT in food intake in situations with high arousal level (Choi et al. 2010). Orexins released in the PVT may act as a modulator in the high-arousal situations associated with food intake (Choi et al. 2012, Kirouac 2015). It was also found that the anticipation of a high-fat food like chocolate increased c-Fos expression of orexin neurons in the hypothalamus as well as OX1R-expressing neurons in the PVT (Choi et al. 2010). Moreover, injections of orein-A in the PVT increased the release of dopamine in the NAc and OX1R knockdown in the PVT reduced the overconsumption of high-fat diet in food deprived rats (Choi et al. 2012).

Similarly, multiple lines of evidence support the role of the PVT in the regulation of drug seeking (Kirouac 2015, Matzeu, Zamora-Martinez, and Martin-Fardon 2014). The expression of c-Fos in neurons in the PVT was elevated by a single exposure to a long list of addictive drugs including cocaine, amphetamine, morphine, nicotine, and alcohol (Barson, Ho, and Leibowitz 2015, Brown, Robertson, and Fibiger 1992, Deutch, Bubser, and Young 1998, Garcia, Brown, and Harlan 1995, Gutstein et al. 1998, Pasumarthi and Fadel 2008, Ryabinin and Wang 1998). Furthermore, PVT neurons have also been shown to be activated by reexposure to cues or contexts which have been paired with addictive drugs (Brown, Robertson, and Fibiger 1992, Dayas et al. 2008, Franklin and Druhan 2000, James et al. 2011, Perry and McNally 2013, Rhodes, Ryabinin, and Crabbe 2005, Wedzony et al. 2003). In contrast, evidence shows that inactivation of the PVT interferes with drug seeking behaviors (Browning, Jansen, and Sorg 2014, Hamlin et al. 2009, James et al. 2010, Marchant, Furlong, and

McNally 2010, Young and Deutch 1998). For instance, lesions of the PVT blocked the conditioned behaviors to the context previously paired with cocaine and prevented the reinstatement of alcohol seeking (Hamlin et al. 2009, James et al. 2010, Marchant, Furlong, and McNally 2010, Young and Deutch 1998). Inactivation of the PVT by the GABA agonists baclofen/muscimol or the sodium channel blocker tetrodotoxin interfered with the expression and reinstatement of cocaine seeking (Browning, Jansen, and Sorg 2014, James et al. 2010). A specific contribution of orexins to drug seeking has recently attracted attention (Barson, Ho, and Leibowitz 2015, Pasumarthi and Fadel 2008). For example, acute systemic administration of nicotine significantly activated the orexin neurons which project to the PVT (Pasumarthi and Fadel 2008). Rats trained to drink ethanol had higher gene expression level of orexins in the hypothalamus and higher mRNA level of OX2R in the anterior PVT (Barson, Ho, and Leibowitz 2015). In addition, ethanol intake was enhanced by microinjections of orexin-A or orexin-B in the anterior PVT and reduced by the OX2R antagonist TCSOX229 (Barson, Ho, and Leibowitz 2015).

The anatomical evidence also points to the possibility that the PVT may participate in modulating behaviors related to fear and anxiety. The PVT provides heavy projections to the dorsolateral BST and capsular and lateral CeA which innervate the areas associated with the modulation of the autonomic and motor responses of fear and anxiety (Dong et al. 2001, Kirouac 2015, Petrovich and Swanson 1997). Emerging evidence appears to support the hypothesis that the PVT may participate in late retrieval of fear memories (days to weeks after conditioning)

(Do Monte et al. 2016). For instance, studies have shown that electrolytic lesions of the posterior PVT or inactivation of the dorsal midline thalamus with muscimol 24 h after the footshocks alleviated the fear response to the auditory cues previously paired with footshocks (Li et al. 2014, Padilla-Coreano, Do-Monte, and Quirk 2012). In addition, our laboratory found that microinjection of the OX2R antagonist TCSOX229 but not the OX1R SB334867 into the posterior PVT blocked the expression of conditioned place aversion induced by naloxone-precipitated morphine withdrawal suggesting that the OX2R in the PVT may be involved in negative emotional state (Li et al. 2011).

Although the mechanisms for PVT mediated fear or anxiety are not known, the pathway for orexin-PVT represents a good candidate. First, orexin neurons in the hypothalamus could be activated by many different stressors (Chang et al. 2007, Furlong et al. 2009, Ida et al. 2000, Sakamoto, Yamada, and Ueta 2004, Winsky-Sommerer et al. 2004). Second, knockout of ppOX or orexin receptors as well as systemic injections of orexin receptor antagonists have been shown to attenuate fear-like or anxiety-like behaviors in different paradigms (Furlong et al. 2009, Kayaba et al. 2003, Soya et al. 2013, Steiner, Lecourt, and Jenck 2012). Third, administration of orexin-A or orexin-B in the PVT elevated the anxiety induced by height and/or open space (Heydendael et al. 2011, Li et al. 2009, Li et al. 2010). For example, injections of orexin-A or orexin-B in the PVT increased the expression of grooming and freezing behaviors in the open field (Li et al. 2009, Li et al. 2010). Injection of orexin-A in the posterior PVT decreased the time spent in the open arms

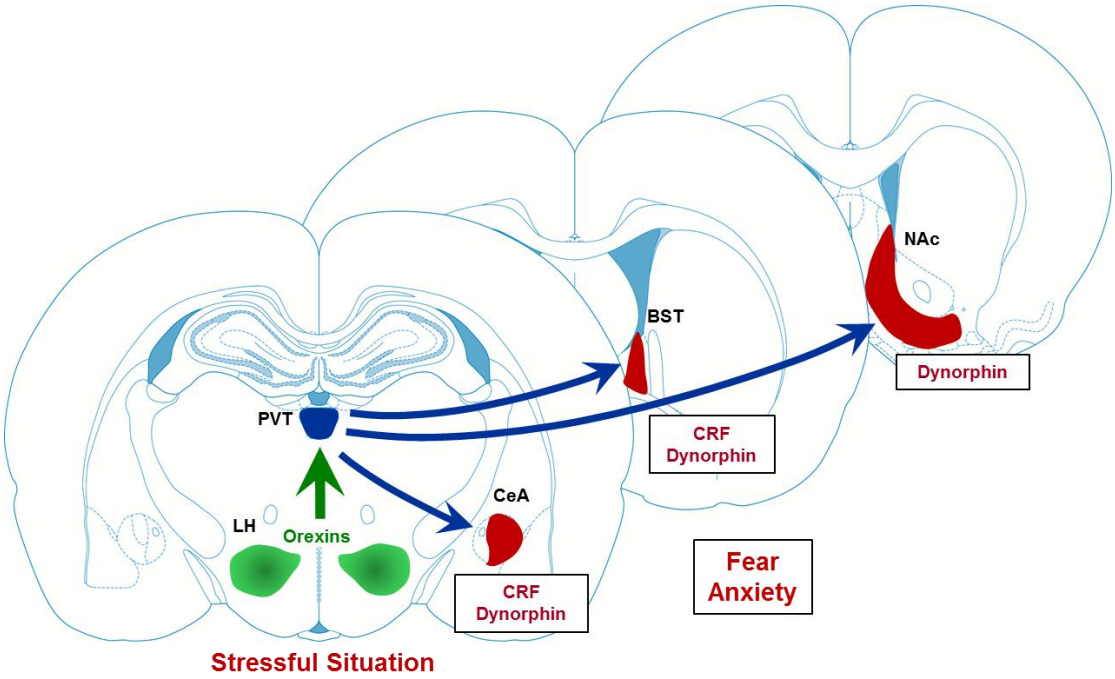
and increased the time spent in the closed arms in the elevated plus maze (Heydendael et al. 2011). Fourth, studies have shown that blockade of orexin receptors in the posterior PVT reduced anxiety levels in rats (Dong, Li, and Kirouac 2015, Heydendael et al. 2011). For example, injection of a dual orexin receptor antagonist called TCS1102 in the posterior PVT decreased the latency in the social interaction test whereas the drug had no effect on freezing behavior when the rats were exposed to auditory cues or context previously paired with footshocks (Dong, Li, and Kirouac 2015). Blockade of OX1R in the posterior PVT with SB334867 elevated the time spent in the open arms and reduced the time spent in the closed arms in the elevated plus maze (Heydendael et al. 2011).

In summary, the unique afferents and efferents of the PVT place this nucleus in a strong position to regulate several motivational and emotional behaviors. Even though the mechanisms are not known, orexins may regulate behaviors like drug seeking, fear and anxiety, through the innervation of the neurons in the PVT.

1.4. Working Hypothesis

As shown in the Fig. 1, the working hypothesis is that the activity of the orexin system is upregulated when rats are exposed to a stressful situation and that the orexin system contributes to anxiety-like and fear-like behavioral states by acting on PVT neurons that modulate CRF and dynorphin neurons in forebrain.

Fig. 1. Schematic illustrating the hypothetical involvement of the PVT as well as CeA, BST and NAc in the orexin mediated fear and anxiety (adapted from Kirouac, 2015). The paraventricular nucleus of the thalamus (PVT) receives a very dense projection from orexin neurons in the lateral hypothalamus (LH). In turn, the neurons in the PVT project to areas of the brain that control fear and anxiety, such as the central nucleus of the amygdala (CeA), the bed nucleus of the stria terminalis (BST) and the nucleus accumbens (NAc). In these brain areas, excitatory projections from the PVT overlap with neurons densely stained for corticotropin-releasing factor (CRF) and dynorphin, both of which play a role in stress related behaviors.


NAc
BST
Dynorphin
PVT
CRF
Dynorphin
CeA
LH
Orexins
CRF
Dynorphin
Fear
Anxiety
Stressful Situation

1.5. Objectives

The present thesis studies the role of the orexin system in anxiety and fear and some of the underlying mechanisms involved. First, experiments were done to characterize whether the PVT was a part of the pathway that the orexins use to regulate anxiety-like behaviors. Second, experiments were done to determine if there were long-lasting changes in the orexin system and other peptide systems that are known to mediate the behavioral responses to aversive situations. A secondary focus of these experiments was to determine if dynorphin, enkephalin and CRF peptides, which are found in high concentrations in regions innervated by the PVT, are associated with orexin-PVT mediated anxiety. Third, a series of pharmacological experiments were done to determine which orexin receptor was involved in contextual fear and avoidance in the footshock model of PTSD.

In summary, the study focused on the role of the orexin system in fear and anxiety and the role of the PVT in this process. In addition, some of the possible mechanisms that are involved in the regulation were identified using behavioral, biochemical, and pharmacological methods. A rat model of PTSD was established to examine the mechanisms of fear and anxiety in stressed animals. In addition, pharmacological experiments were employed to examine the specific role of orexin receptors in distinct behaviors. Biochemical methods including real time reverse transcription-polymerase chain reaction (RT-PCR) and western blot were employed to examine the changes of orexin system and other stress-related peptide systems including CRF, dynorphin and enkephalin after footshock exposure.

1.5.1. Objective 1: To Characterize the Role of Orexins on Regulating Anxiety by Acting on the PVT

As discussed in the previous section, the PVT provides inputs to parts of the extended amygdala, the prefrontal cortex and basolateral amygdala (Hsu and Price 2009, Li and Kirouac 2008, Parsons, Li, and Kirouac 2007, Vertes and Hoover 2008). The medial prefrontal cortex and the basolateral amygdala, along with the parts of the extended amygdala, represent an important cortical-subcortical circuit involved in emotional behaviors (Cardinal et al. 2002, Walker, Toufexis, and Davis 2003), all of which can be influenced by the PVT. In addition, orexin neurons provide extensive projections to the PVT and this region of thalamus represents a likely area of the brain involved in emotions and the regulation of behavioral arousal associated with a stressful experience (Mahler et al. 2012, Sakurai 2014, Sakurai et al. 1998). Thus, ***the hypothesis that orexins in the PVT modulate anxiety was tested***. In addition, afferents from the PVT overlap with subregions of the extended amygdala that contain neurons densely stained for dynorphin and CRF (Li and Kirouac 2008), two peptides strongly implicated in the expression of negative emotional state (Davis 1998, Davis et al. 2010, Heinrichs and Koob 2004, Shirayama and Chaki 2006). To further study the mechanisms underlying orexin-inducing anxiety, ***the hypothesis that the CRF antagonist and kappa opioid receptor antagonist could block the anxiety induced by orexin-A injections in the PVT was tested.***

1.5.2. Objective 2: To Characterize Changes in the Orexin, Dynorphin, Enkephalin and CRF Systems in a Footshock Model of PTSD

Experimental evidence indicates that orexins stabilize arousal during periods of wakefulness (Berridge, Espana, and Vittoz 2010, Boutrel, Cannella, and de Lecea 2010, Sakurai 2007, Taheri, Zeitzer, and Mignot 2002) and that loss of orexin signaling causes difficulty in maintaining wakefulness (Sakurai 2007, Taheri, Zeitzer, and Mignot 2002). Other studies showed that orexin neurons are activated in response to stressful conditions (Espana, Valentino, and Berridge 2003, Furlong et al. 2009, Ida et al. 2000, Winsky-Sommerer et al. 2004, Zhu et al. 2002) and play a role in the physiological and behavioral responses to stress (Furlong et al. 2009, Kayaba et al. 2003, Zhang et al. 2010). Therefore, orexins may also be involved in the regulation of anxiety disorders, such as the PTSD. Here the study examined the long-lasting changes in the orexin system in a footshock model of PTSD. ***The hypothesis that there is an upregulation of the orexin system in rats exposed to footshocks was tested.***

Furthermore, previous work in our laboratory showed that a subgroup of shocked rats exhibited high anxiety level (HR) while the other rats that received footshocks showed similar anxiety level to the nonshocked rats (LR) (Chen et al. 2012). The individual difference in anxiety that develops in rats exposed to footshocks may be due to the changes of the orexin system. So the study further examined the long-lasting changes of orexin synthesis in HR, LR and nonshocked rats. ***The hypothesis that ppOX mRNA level is higher in HR rats compare to LR and***

nonshocked rats at 14 days after the footshock exposure was tested.

Experimental evidence from a variety of sources indicates that some parts of the extended amygdala are involved in the behavioral reaction to stress and negative emotional states such as dysphoria, fear and anxiety (Davis et al. 2010, Koob 2003). The lateral portion of the BST and the lateral subnucleus of the CeA contain neurons that produce CRF (Cassell, Freedman, and Shi 1999), a peptide showed to mediate the behavioral effects of stress (Heinrichs and Koob 2004). The opioid peptide dynorphin is another stress-related peptide found in high concentration in the extended amygdala and the shell of the NAc (Cassell, Freedman, and Shi 1999, Cassell, Gray, and Kiss 1986, Furuta, Zhou, and Kaneko 2002, Poulin et al. 2009). A number of observations implicate dynorphin in the NAc in the behavioral responses to stress (Knoll and Carlezon 2010). Other studies indicate that decrease in enkephalin activity in the amygdala contribute to anxiety-like behavior following acute and chronic stress and that the activity of these enkephalin neurons promote individual resilience to stressful situations (Berube et al. 2013, Berube et al. 2014, Hebb et al. 2004). Therefore, experiments were done to examine long-lasting changes of the production of CRF, dynorphin and enkephalin in related brain areas including BST, CeA, caudate putamen, and NAc after footshocks. ***The hypothesis that the CRF and dynorphin systems are enhanced in the HR following footshock exposure was tested.***

1.5.3. Objective 3: To Characterize the Specific Subtype of Orexin Receptors Involved in Contextual Fear and Anxiety in a Rat Model of PTSD

Studies showed that administration of dual orexin receptor antagonists reduced fear-induced startle (Steiner, Lecourt, and Jenck 2012) and attenuated the increases in heart rate and blood pressure produced by exposing rats to the chamber in which they had previously experienced footshocks (Furlong et al. 2009). The receptor subtype mediated these effects has not been identified with pharmacological studies. Accordingly, ***the hypothesis that blocking orexin receptors with a nonspecific antagonist (TCS1102) and the specific OX1R antagonist (SB334867) and OX2R antagonist (TCSOX229) attenuates fear and anxiety in shocked rats was tested.***

Chapter 2

Materials and Methods

2.1. Animals and Housing

Male Sprague-Dawley rats of 6 weeks of age (University of Manitoba, Winnipeg, Manitoba, Canada) were pair-housed in plastic cages in a colony room on a 12-hour light-dark cycle (lights on 6 am) with controlled temperature (20-24 °C) and humidity (40-70 %). All of the rats had free access to food and water. In order to habituate the animals to the experimenters, rats were handled every other day before the first experimental procedure in a 12 day adaptation period. The handling procedure reduces the anxiety induced by handling by the experimenters and allows the behavioral tests to properly evaluate experimental induced anxiety and fear. All experimental procedures were conducted during the light cycle. The experimental protocols were approved by Research Ethics Review Board of University of Manitoba and in compliance with the Canadian Council on Animal Care.

2.2. Animal Models of Anxiety, Fear and PTSD

There are remarkable similarities between human and mammals in terms of how the brain controls emotions like fear and anxiety (Parmigiani et al. 1999). Animal models are widely used in all aspects of biomedical research for several reasons (Borghans and Homberg 2015, van der Staay 2006, van der Staay FJ 2009, Yehuda and Antelman 1993). First, animal subjects are tested under controlled conditions

which simplify the complex human conditions and reveal the relationship between specific target factors and behaviors. Second, compared to human subjects, it's easier to have enough animals in experiments to reach statistical reliability. Third, investigators can induce symptoms that resemble a clinical disorder by using a variety of methods and then manipulate some factors in the development of the symptoms to better understand the underlying mechanism of the clinical disorder. Fourth, animal models are used to assess effect and risk of various new treatments and drugs.

Several animal models have been developed to study the underlying mechanism and potential treatment of PTSD (Borghans and Homberg 2015, Daskalakis, Yehuda, and Diamond 2013, Whitaker, Gilpin, and Edwards 2014). Current animal models for PTSD usually involve exposing rodents to a variety of stressful conditions including physical, social, and psychological stressors (Borghans and Homberg 2015, Daskalakis, Yehuda, and Diamond 2013, Whitaker, Gilpin, and Edwards 2014). Physical stressors are relatively basic strategies to mimic PTSD-inducing trauma. Typical physical stressors include single or repeated episodes of electrical footshock, restraint stress and single prolonged stress (Chen et al. 2012, Grafe et al. 2016, Khan and Liberzon 2004, Louvart et al. 2005, Louvart et al. 2006). Social stressors include social defeat, social isolation/maternal separation and housing instability (Butler, Ariwodola, and Weiner 2014, Hammels et al. 2015, Saavedra-Rodriguez and Feig 2013, Ver Hoeve et al. 2013). Moreover, predator or predator odor is used as psychological stressors (Adamec, Blundell, and Burton 2005, Cohen and Zohar 2004, Fifield et al. 2013). These animal models of PTSD produce a number of PTSD-like

symptoms, such as heightened anxiety, elevation of arousal, and enhanced fear (Adamec, Blundell, and Burton 2005, Borghans and Homberg 2015, Butler, Ariwodola, and Weiner 2014, Chen et al. 2012, Cohen and Zohar 2004, Fifield et al. 2013, Grafe et al. 2016, Hammels et al. 2015, Khan and Liberzon 2004, Louvart et al. 2005, Louvart et al. 2006, Saavedra-Rodriguez and Feig 2013, Ver Hoeve et al. 2013, Whitaker, Gilpin, and Edwards 2014). Several models also induce dysfunction of the HPA axis and the autonomic system (Borghans and Homberg 2015, Butler, Ariwodola, and Weiner 2014, Cohen and Zohar 2004, Grafe et al. 2016, Hammels et al. 2015, Khan and Liberzon 2004, Louvart et al. 2005, Louvart et al. 2006, Ver Hoeve et al. 2013, Whitaker, Gilpin, and Edwards 2014).

One of the most reliable animal models for PTSD is exposing rodents to an episode of inescapable and relatively intense footshocks (Chen et al. 2012, Louvart et al. 2005, Louvart et al. 2006). The footshock exposure, which is similar as a severe traumatic event to people, leads to a strong fear response in rodents. This paradigm produces several behavioral changes which can last more than 1 month (Chen et al. 2012, Louvart et al. 2005, Louvart et al. 2006). Rodents exposed to footshock show increased anxiety as evidenced by enhanced avoidance to novel environments and contexts/cues related to the footshock, reduced social behavior, elevated fear to shock context and novel environment, and increased acoustic startle response (Bruijnzeel, Stam, and Wiegant 2001b, a, Louvart et al. 2005, Louvart et al. 2006, Mikics, Baranyi, and Haller 2008, Mikics et al. 2008, Pynoos et al. 1996, Siegmund and Wotjak 2007, van Dijken, Mos, et al. 1992, Van Dijken, Van der Heyden, et al. 1992). In addition,

footshock exposure induces a disturbance of plasma corticosterone feedback to stress as well as dysfunction of HPA axis and the autonomic system in rodents representing classic PTSD responses in human (Bruijnzeel, Stam, and Wiegant 2001a, Daskalakis, Lehrner, and Yehuda 2013, Louvart et al. 2005, Louvart et al. 2006, Mikics et al. 2008).

The intensity of stress and the degree of exposure in this paradigm can be carefully controlled by adjusting the intensity and duration of the footshock. Some symptoms of PTSD are operationally defined by using the behavioral response of rodents in different experimental tests (Chen et al. 2012, Goswami et al. 2013). Various mazes including the elevated plus maze and elevated T-maze have been employed to evaluate the anxiety-like and avoidance behaviors induced by footshock exposure (de Paula Soares et al. 2011, Louvart et al. 2005, Pynoos et al. 1996). The elevated plus maze consists of two elevated open alleys (open arms) and two enclosed alleys (closed arms) which are arranged to form a plus shape (Walf and Frye 2007). This paradigm produces a strong approach-avoidance conflict. That is the approach of rodents' proclivity towards dark enclosed spaces and the avoidance of heights and open spaces (Walf and Frye 2007). An anxious rat always tends to stay in the safety of the closed arms and does not explore the open arms as much as a non-anxious rat. The anxiety behavior of rodents in the elevated plus maze is usually assessed by using the ratio of time spent in the open arms to the total time spent in the open arms and closed arms (percent open arm time) (Pellow et al. 1985, Rodgers and Johnson 1995). Lower percent open arm time is recognized as higher level of anxiety. In addition,

the expression of ethological behaviors in this paradigm is also helpful for assessing the rodent's anxiety level (Carobrez and Bertoglio 2005, Hogg 1996, Rodgers et al. 1997). Various ethological behaviors reveal distinct aspects of anxiety that can be used to confirm the presence of an anxious state demonstrated by the time spent in the open and closed arms. Similarly, the elevated T-maze is similar to the elevated plus maze and is used to mainly measure the anxiety by assessing the avoidance of heights and open spaces (Graeff, Netto, and Zangrossi 1998, Zangrossi and Graeff 1997).

Moreover, defensive withdrawal and a variety of shuttle boxes are used to assess avoidance to novel environment and context/cues related to the footshock (Bruijnzeel, Stam, and Wiegant 2001b, a, Chen et al. 2012, Louvart et al. 2005, Louvart et al. 2006). An anxious rat tends to stay in the dark safe chamber and avoids exploring novel spaces/chambers. In addition, several studies have examined the changes in social behavior induced by footshock exposure using social interaction test (also called social avoidance test) (Chen et al. 2012, Mikics, Baranyi, and Haller 2008, Mikics et al. 2008). A rat with high anxiety level will not interact with an unfamiliar rat whereas a non-anxious rat will. One of the most typical symptoms of PTSD is the fear that can be triggered by reminders of the trauma (Careaga, Girardi, and Suchecki 2016). Fear conditioning is a good model to measure the conditioned fear response to context or cues related to trauma (Chen, Li, and Kirouac 2014, Chen et al. 2012, Soya et al. 2013). The open field is employed to test the generalized fear of a novel and open environment (Chen et al. 2012, van Dijken, Mos, et al. 1992, Van Dijken, Van der Heyden, et al. 1992). Several laboratories also measure acoustic

startle response to represent as a parameter of hyperarousal or hypervigilance in rodents (Cohen et al. 2012, Pynoos et al. 1996). In addition, one of the advantages of animal models is that it is possible to measure changes in distinct neurochemical systems as anxiety-like behavioral responses develop following exposure of animals to stressful conditions. A number of molecular methods, such as radioimmunoassay, immunohistochemistry and in situ hybridization, can be used to reveal the mechanism behind the development of PTSD (Louvart et al. 2005, Louvart et al. 2006, Mikics et al. 2008).

As mentioned earlier, people exposed to a life-threatening event will likely exhibit various symptoms like intense nightmares, anxiety, insomnia and numbing. Only a small proportion of these individuals will develop PTSD whereas these symptoms will dissipate over time in other individuals (Levin, Kleinman, and Adler 2014, Warner et al. 2013). Consequently, it is essential to identify individual differences in the development of PTSD. It is recognized that the acute response to a traumatic event (the peritraumatic period) may help to predict the future diagnosis of PTSD (Cardena and Carlson 2011). Indeed, studies have revealed the plausible relationship between the ASD and the PTSD (Bryant and Harvey 1998, Bryant et al. 2000, Harvey and Bryant 1998). People with ASD have a much higher probability of being diagnosed with PTSD several months later (Bryant and Harvey 1998, Bryant et al. 2000, Harvey and Bryant 1998). A state of hyperarousal is one of the major symptoms in the ASD and the PTSD and may play a critical role in the development of other PTSD symptoms (Cardena and Carlson 2011). It has been shown that an

intense hyperaroused state early after a trauma experience is a good predictor of the development of other PTSD symptoms (Marshall et al. 2006, Schell, Marshall, and Jaycox 2004, Solomon, Horesh, and Ein-Dor 2009, Thompson et al. 2004, Weems et al. 2003).

2.3. Effects of Orexins Microinjections in the PVT on Anxiety Response

2.3.1. Cannula Implantation

Rats were anesthetized with equithesin (0.3 ml/100 g, intraperitoneal (i.p.)) and placed in a Stoelting stereotaxic frame. The rat's head was shaved and an incision was made to expose the skull. Then a hole in the skull was made at the selected point using a specialized bone drill. A stainless steel guide cannula (23 gauge, Plastics One, VA, USA) was unilaterally implanted into the posterior aspect of the PVT (3.1 mm posterior to bregma, 1.3 mm lateral to the midline, and 4.0 mm ventral to the skull, angle at 10°, with the incisor bar at 3.3 mm below intraaural line). Another subgroup of rats had a single guide cannula implanted in the lateral thalamus (3.1 mm posterior to bregma, 3.0 mm lateral to the midline, and 4.3 mm ventral to the skull). Stainless steel screws and dental cement were used to secure the guide cannula in place. A capped stylet (Plastics One, VA, USA) was inserted to prevent occlusion of the guide cannula and all rats were treated with penicillin (80,000 units) after surgery. Rats were allowed to recover for 10-14 days, during which they were handled every other day to reduce stress associated with handling at the time of testing.

2.3.2. Drugs and Microinjections

The orexin-A and orexin-B peptides (Tocris, UK) were dissolved in saline. Drug solutions were stored in aliquots at -20 °C and thawed immediately before the microinjections. A mock microinjection was given to the rats each day in the test room for 3 days before the actual experiments to habituate the animals to the injection procedure. In the mock microinjection, an injector cannula (30 gauge, Plastics One, USA) was inserted into the guide cannula and held for 2 min but no drug was delivered. Before the elevated plus maze test, the drugs or vehicle (0.5 μl for each injection) was injected through an injector cannula which protruded 2.0 mm below the guide cannula. Infusions were delivered with a Hamilton micro-syringe mounted on a motorized pump (Stoelting Co, IL, USA). The injection was done at the rate of 0.25 μl/min over 2 min. The stylet was inserted back into the guide cannula and the rat was returned to its home cage for 5 min before the test.

After the recovery from the surgery, subgroups of rats received one of three different concentrations of orexin-A (0.3 μg, 3.0 μg and 10.0 μg), orexin-B (3.0 μg) or the vehicle (saline) in the PVT 5 min prior to the elevated plus maze test. A subgroup of rats was tested in the elevated plus maze following microinjections of orexin-A (3.0 μg) in the lateral thalamus.

2.3.3. Elevated Plus Maze Test

The elevated plus maze (Med Associates, VT, USA) was composed of black Plexiglass and consisted of two open arms (length 50 cm × width 10 cm × height 0.5

cm) and two closed arms (length 50 cm × width 10 cm × height 40 cm) with all the arms extended from a center area (length 10 cm × width 10 cm). The maze was elevated 50 cm above the floor and placed in a dimly lit room with two 15 W bulbs. The illumination in open and closed arms was between 2.0-5.0 lx and less than 0.5 lx, respectively.

With a few modifications, the elevated plus maze test was conducted as described previously (Walf and Frye 2007). Briefly, a rat was placed in the center area of the elevated plus maze facing one of the closed arms and the movement of the rat was tracked by infrared sensors and a video camera for 5 min. After each test, the maze was cleaned with 2.0 % ethanol and dried to prevent interference of olfactory cues in subsequent tests. The time spent as well as the number of entries in open and closed arms, was recorded using software (Med Associates, VT, USA). The data were converted into the percent open arm time (time in open arms/time in open arms + time in closed arms) and percent open arm entry (number of entries to open arm/number of entries to open arm + number of entries to closed arms), both of which represent indices of anxiety (Pellow et al. 1985, Rodgers and Johnson 1995). The number of entries into the closed arms was used as a measure of locomotor activity as previously done (Cruz, Frei, and Graeff 1994, Espejo 1997, File 2001).

The expression of ethological behaviors in the elevated plus maze is also useful for assessing anxiety level (Carobrez and Bertoglio 2005, Hogg 1996, Rodgers et al. 1997). The following behavioral variables were quantified from video records of the elevated plus maze test: (1) latency to enter the open arms (open arm latency); (2)

duration of head scanning outside of a closed arm with part of the body remaining in the closed arms (scanning time); (3) number of episodes where body and head are stretched forward (stretch-attend postures, SAP); (4) time spent exploring the end of the open arms (end arm time); (5) time spent dipping the head down from the open arms and center area (head dipping time); (6) duration of grooming in the closed arms (grooming time); and (7) number of defecations (defecation number). The behavioral analysis was done by two observers blind to the group identity of the subjects (the correlation coefficient for two observers ranged from 0.91-0.97).

2.3.4. Cannula Verification

Rats were deeply anesthetized with chloral hydrate (40 mg/kg) and perfused transcardially. The rat's chest cavity was opened with surgical scissors. Then a metal needle connected to a peristaltic pump was inserted into the left ventricle with the needle tip in the ascending aorta. The rats were perfused with heparinized saline followed by 4.0 % paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). After that, brains were removed and placed in beakers with 4.0 % paraformaldehyde for 4 hours at 4 °C. The brains were transfered into 20 % sucrose solution and kept in this solution at 4 °C until the brains sank to the bottom. Then the brains were transfered into 30 % sucrose solution and kept in this solution at 4 °C until the brains sank to the bottom. After that, a vibratome was used to obtain 100 μm coronal sections from the injection site (Paxinos and Watson 2009). The locations of the cannula tips were verified in relation to the PVT region on brain sections stained

immunohistochemically for orexin-A as previously done (Kirouac, Parsons, and Li 2005). Rats with cannula placements that were more than 0.5 mm away from the PVT were excluded from the analysis.

2.3.5. Statistical Analysis

The locations of the cannula tips were verified on brain sections stained immunohistochemically for orexin-A as done previously and described in section 2.3.4 (Kirouac, Parsons, and Li 2005). All data were analyzed using one-way analysis of variance (ANOVA) and with post hoc Dunnett analysis to determine if differences between groups were significant.

2.4. Effects of CRF and Kappa Opioid Receptor Blockade on Anxiety Produced by Orexin-A Microinjection in the PVT

2.4.1. Cannula Implantation

Two guide cannulae were implanted as previously described in section 2.2.1. Briefly, rats were anesthetized with equithesin (0.3 ml/100 g, i.p.) and placed in a Stoelting stereotaxic frame. A stainless steel guide cannula (23 gauge, Plastics One, VA, USA) was unilaterally implanted into the posterior aspect of the PVT (3.1 mm posterior to bregma, 1.3 mm lateral to the midline, and 4.0 mm ventral to the skull, angle at 10°, with the incisor bar at 3.3 mm below intraaural line). A second cannula was implanted in the lateral ventricle (0.8 mm posterior to bregma, 1.4 mm lateral to midline, and 2.5 mm ventral to the skull).

After the surgery, rats were allowed to recover for 10-14 days, during which they were handled every other day to reduce stress associated with handling at the time of behavioral testing.

2.4.2. Drugs and Microinjections

The orexin-A peptide (Tocris, UK), the non-selective CRF antagonist hCRF (Tocris), and the kappa receptor antagonist norbinaltorphimine (nBNI; Tocris) were dissolved in saline. Drug solutions were stored in aliquots at -20 °C and thawed immediately before the microinjections. The microinjections were done as previously described in section 2.3.2. In this experiment, the volume of each injection is 0.5 μl for the PVT and 2.0 μl for the lateral ventricle.

After the recovery of surgery, the CRF antagonist hCRF (1.0 μg) was infused in the lateral ventricles 30 min before receiving a microinjection of orexin-A (3.0 μg) in the PVT. Since there is a debate as to when nBNI should be given to completely block the kappa opioid receptors (Knoll et al. 2007, Land et al. 2008), injections of nBNI (10.0 μg) in the lateral ventricles at both 24 h and 30 min before the orexin-A microinjections were chose. Another experiment in which hCRF, nBNI, or saline was given in the lateral ventricles and saline in the PVT region was done to determine if the CRF and kappa opioid receptor antagonists produce nonspecific effects on behaviors on the elevated plus maze.

2.4.3. Elevated Plus Maze Test

After receiving different drugs, the rats were tested in the elevated plus maze as described in section 2.3.3. In this experiment, the time spent in the open arms and closed arms, the number of entries into the closed arms and the expression of ethological behaviors (open arm latency, scanning time, number of SAP, end arm time, head dipping time, grooming time, defecation number) were analyzed for each rat by two observers who were blinded to group assignment.

2.4.4. Cannula Verification

The locations of the cannula tips were verified as described in section 2.3.4 (Kirouac, Parsons, and Li 2005). Briefly, rats were deeply anesthetized and perfused transcardially. Coronal sections with the PVT were obtained and stained immunohistochemically for orexin-A. Rats with cannula placements that were more than 0.5 mm away from the PVT were excluded from the analysis.

2.4.5. Statistical Analysis

One-way ANOVA was used to analyze all the data with post hoc Dunnett analysis to determine if differences between groups were significant.

2.5. Long-Lasting Changes of the Orexin System in Rats Exposed to Footshocks

2.5.1. Footshock Procedure

The rats were adapted to the experimenters and the testing rooms prior to shock

procedures. The rats were transferred one at a time to a testing room (400-500 lx) which was exclusively dedicated for footshock delivery in an inescapable footshock chamber (MED Associates, St. Albans, Vermont, USA). After a 2 min acclimation period, rats were exposed to footshocks (5 × 2 s of 1.5 mA presented over 3 min) (Chen et al. 2012). The footshocks were presented randomly with an intershock periods of 10-50 s which was controlled by a computer program. After the last footshock, the rats were kept in the footshock chamber for 60 s and then they were returned to their home cages. Nonshocked rats were placed in the footshock chamber for the same amount of time but no footshock was delivered. The footshock chamber was cleaned with 10% alcohol and the bedding was replaced after exposure of each rat. There was no other behavioral test prior to being sacrificed.

2.5.2. Tissue Preparation

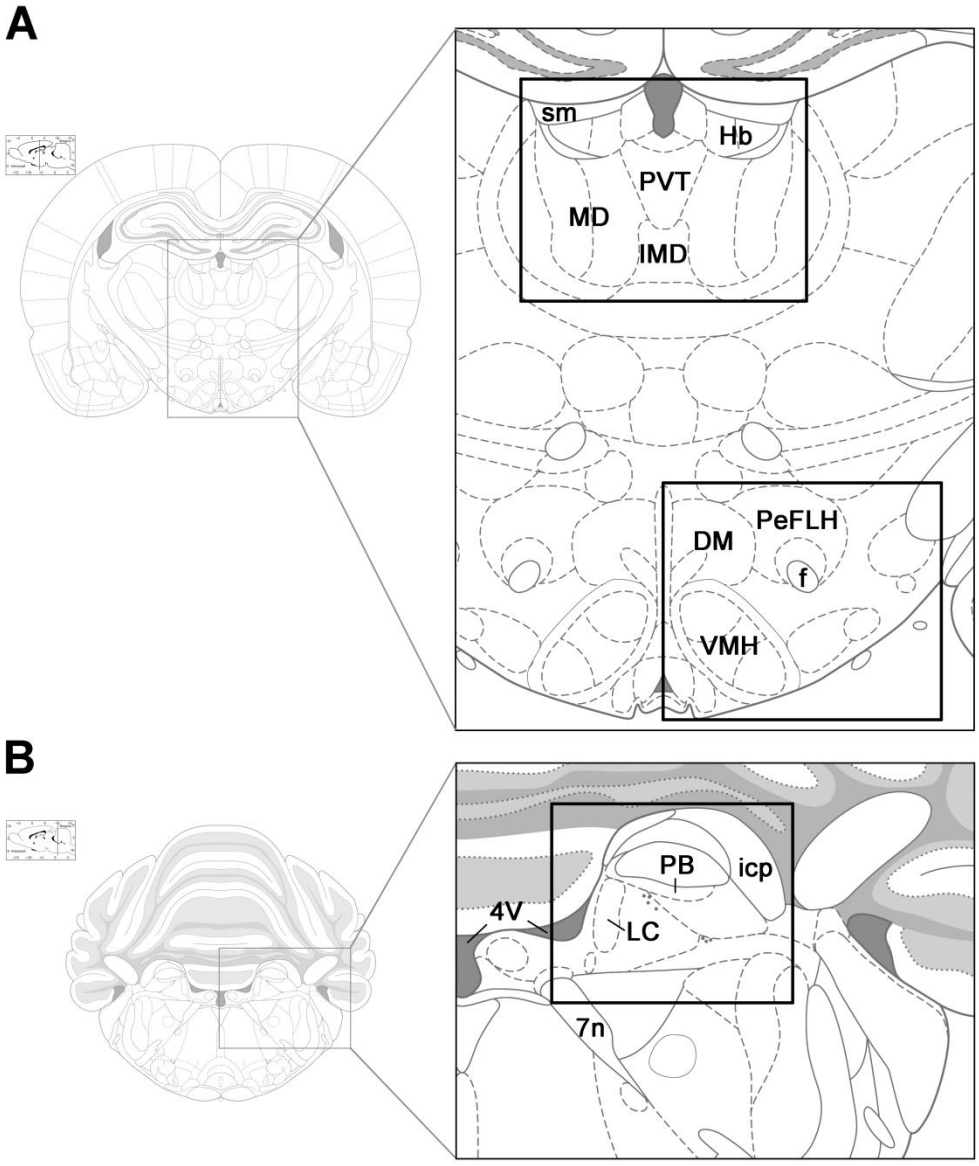
Selected brain areas of interest were preserved for subsequent analysis. At 11 days post-shock, rats were anaesthetized using chloral hydrate (600 mg/kg, i.p.) and perfused transcardially with ice-cold 0.1M phosphate buffered saline (PBS) as described in section 2.3.4. After that, brains were removed and placed in a matrix with 1 mm divisions to cut sections of selected brain areas. The coronal sections were placed on a plastic petri dish and areas of interest were dissected out using a dissecting microscope according to an anatomical atlas (Paxinos and Watson 2009). The hypothalamus (Fig. 2A), midline thalamus (Fig. 2A) and locus coeruleus/parabrachial regions (Fig. 2B) were dissected and stored at -80 °C for later

analysis using RT-PCR. Hypothalamic samples from the opposite side of the brain were used to do western blot analysis of OX1R and OX2R. All procedures were done on ice with all surfaces in contact with the brain tissue cleaned with RNaseZap (Life Technologies Inc., ON, Canada). Tissues were collected in RNase-free microtubes containing RNAlater solution (Life Technologies Inc., ON, Canada) and stored at -80 °C for later analysis RT-PCR. Brain samples collected for western blot analysis were preserved in microtubes and frozen immediately with dry ice and stored at -80 °C.

2.5.3. RT-PCR

RT-PCR was done to quantify the mRNA levels of ppOX, OX1R and OX2R in different brain regions. Total RNA was extracted from individual homogenized samples using the protocol in the QIAzol® handbook (Qiagen, ON, Canada) and purified using RNeasy Mini Kit (Qiagen, ON, Canada). The concentration of purified RNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Delaware, USA). The first-strand cDNA synthesis was performed using qScript™ cDNA SuperMix (Quanta BioSciences, Maryland, USA). Quantitative PCR was performed on the genes of interest in triplicate using an Eco™ Real-Time PCR system (Illumina, California, USA). All PCR reactions were carried out in a PCR MasterMix consisting of 5.1 µl of RNase-free water, 0.9 µl of each primer mix (FWD + REV; 20 µM), 2.0 µl of cDNA, and 2.0 µl of MBI EVOlution EvaGreen® qPCR master mix (MBI Lab Equipment, PQ, Canada).

Fig. 2. Diagrammatic representation of the regions of the brain dissected for analysis of mRNA levels for prepro-orexin and orexin receptors using real time-PCR (adapted from Paxinos and Watson, 2009). Coronal section A was blocked between -1.0 to -4.0 mm from bregma while coronal section B was blocked between -8.5 to -10.5 mm from bregma. Abbreviations (A) DM: dorsomedial hypothalamic nucleus; f: fornix; Hb: habenula; IMD: intermediodorsal nucleus; MD: medial dorsal nucleus; PeFLH: perifornical lateral hypothalamus; PVT: paraventricular nucleus of the thalamus; sm: stria medullaris; VMH: ventromedial hypothalamus; (B) 4V: 4th ventricle; 7n: facial nerve; icp: inferior cerebellar peduncle; LC: locus coeruleus; PB: parabrachial.


A
sm
Hb
PVT
MD
IMD
PeFLH
DM
f
VMH
B
PB
icp
4V
LC
7n

An amplification and detection procedure was carried out which consists of denaturation at 95°C for 15 min, followed by 40 cycles of 95°C for 10 s, 60°C for 15 s, and 72°C for 15 s, followed by melt curve stage of 95°C for 15 s, 55°C for 15 s and 95°C for 15 s. All PCR data was collected and analyzed using an Eco^{TM} software (Illumina, California, USA) and the relative mRNA level of each selected gene was quantitated using $2^{-\Delta\Delta Ct}$ method. All primers were obtained from Integrated DNA Technologies and validated. The housekeeping gene hypoxanthine phosphoribosyltransferase 1 (Hprt1) was used as the internal quantitative control and all primers were validated. The Hprt1 is a validated house-keeping gene for RT-PCR in the brain (Julian et al. 2014) as indicated by pretest efficiency tests in our laboratory. The primers used were: Hprt-1 forward, 5'- CTC ATG GAC TGA TTA TGG ACA GGA C -3' and reverse, 5'- GCA GGT CAG CAA AGA ACT TAT AGC C -3'; ppOX forward, 5'- CGT CTT CTA TCC CTG TCC TAG T -3' and reverse, 5'- ACA CCA ACA GAG AAA CGT CTT TA -3'; OX1R forward, 5'- GGC AGA TGA ACT CTA CCC TAA G -3' and reverse, 5'- TGC GGA AGA TCT GGA AAT AGG -3'; OX2R forward, 5'- GAG ATA AAG CAG ATC CGA GCA C -3', and reverse, 5'- TGT GAA CTA CCC GAA CAC TC -3'. For validation of the primers, a default melting curve program was run after each PCR cycling program to make sure that disassociation curves for each pair of primers contained a single peak, and the agarose gels of the amplified product revealed single band corresponding to the predictable amplicon length. A calibration curve was performed prior to the initiation of the experiments to determine amplification efficiency.

2.5.4. Western Blot

Brain samples were homogenized using a Potter-Elvehjem tissue grinder (3 ml; Corning, New York, USA) at 4 °C with 1 ml solution A containing 1 μg BSA, 320 mM sucrose, 10 mM HEPES (pH 7.4), 1 mM EGTA, 1 mM $NaHCO_3$, 10 mM sodium pyrophosphate, 1 mM PMSF, and 1 mM sodium orthovanadate. The homogenate was transferred to a new tube and centrifuged at 800 *g* for 20 min at 4 °C twice. The supernatant was transferred to a tube for ultracentrifuge (Beckman Coulter, Ontario, Canada) and centrifuged at 15,000 *g* for 45 min at 4 °C. Then the pellet was re-suspended with solution B containing 1 mM PMSF, and 1 mM sodium orthovanadate in TBS buffer. The total amount of proteins in the obtained suspension was determined by the Bio-Rad DC protein assay.

Samples containing equal amount of proteins (120 μg) were boiled for 6 min in 5 × loading buffer (250 mM Tris-HCl pH 6.8, 80 mg/ml SDS, 40 % glycerol, 1 mg/ml bromophenol blue and 100 mM DTT), run on a 12 % SDS/polyacrylamide gel, and transferred to PVDF membranes using a Mini transblot apparatus (Bio-Rad Laboratories, Pennsylvania, USA). After the transfer procedure, 5 % skim milk in TBST buffer (150 mM NaCl, 20 mM Tris-HCl pH 7.5, 0.1 % Tween 20) was used to block the membranes for 1 h at room temperature. The membranes were incubated overnight with an anti-OX2R antibody (ab183072; Abcam, Massachusetts, USA) at a concentration of 1:1000 at 4 °C. After being washed three times with TBST buffer, the membranes were incubated with horseradish peroxidase conjugated anti-rabbit IgG in a concentration of 1:2500 at room temperature for 1 h. Finally, the proteins

were visualized by ECL western blotting detection reagents (GE Healthcare, Buckinghamshire, UK) on a Fusion FX imaging system (MBI Lab Equipment, Quebec, Canada) and the images of the bands obtained were saved. Bio1D software was used to measure the intensity of bands which represents the relative quantity of the selected protein. To detect the OX1R, the membranes were washed and incubated at room temperature for 5 min in stripping buffer containing 2 % SDS, and 3.8 % glycine, pH 2.0, and re-probed using an OX1R antibody (ab68718; Abcam, Massachusetts, USA) at a concentration of 1:1000 with the same protocol. For β-actin, the membranes were incubated in anti-β actin antibody (ab49900; Abcam, Massachusetts, USA) at a concentration of 1:5000 at room temperature for 1 h and visualized. The orexin receptor antibodies were polyclonal and produced in rabbits and detected a single band at 48 kDa for the OX1R and 51 kDa for the OX2R in immunoprecipitation studies as reported by the supplier.

2.5.5. Statistical Analysis

Group differences were analyzed using a Student's t-test. Note that some samples were lost during processing resulting in variable numbers of cases for the different biochemical tests.

2.6. Long-Lasting Changes of the ppOX mRNA Level in a Modified Footshock Model of PTSD

2.6.1. Modified Footshock Model of PTSD

Our laboratory has modified the footshock model by using a behavioral indicator of hyperarousal during the peritraumatic period as a predictor of the anxiety and avoidance that develop following exposure of rats to the footshock (Chen et al. 2012). In this modified footshock model, rats are exposed to an episode of inescapable footshocks (5 × 2 s of 1.5 mA) and then the acute fear response to a tone is assessed in a novel chamber on the day after the footshock (Chen et al. 2012). The amount of freezing, a classic behavioral index of fear in rodents, is used to show the fear levels of rodents exposed to footshocks (Blanchard and Blanchard 1988). Based on the percentage of the total amount of time spent on freezing, shocked rats are assigned to low responders (LR; freezing% < 40 %) and high responders (HR; freezing% > 60 %). The HR show long-lasting (at least one month after the footshock) and high levels of avoidance of open spaces and novel rats in addition to high levels of fear to the shock context (Chen et al. 2012). In contrast, the LR show similar levels of avoidance as nonshocked rats but high levels of fear to the shock context similar to the HR (Chen et al. 2012). In addition, the HR were found to emit ultrasonic vocalization in a range associated with a negative emotional state (20-30 kHz) when placed in the shock context (Brudzynski 2001, Chen et al. 2012, Litvin, Blanchard, and Blanchard 2007).

The modified footshock approach described above is a good model to study the potential role of hyperarousal in PTSD, because it involves a relatively homogenous

population of rats (same age and sex) with identical life experience (housed as a group) exposed to standardized experimental conditions and tests. Modulators of arousal in the brain may be potential targets for treatment of PTSD because of the potential contributing effects of hyperarousal on other PTSD symptoms. This modified model could further study the changes of these modulators including orexin, CRF, dynorphin and enkephalin, in the individuals that showed anxiety-like and fear-like behaviors after the exposure to footshocks.

2.6.2. Footshock Procedure

Rats received an episode of unpredictable footshocks (5 × 2 s of 1.5 mA) as previously described in section 2.5.1. Nonshocked rats were placed in the chamber for the same amount of time without any footshock. The timeline of the present experiment is shown on Table 1.

2.6.3. Acute Fear Response to a Novel Tone

Acute fear response to a novel tone was assessed on the day after the footshock exposure in a novel chamber (Plexiglas, length 65 cm × width 40 cm × height 50 cm) with a dim light (3-5 lx). The time spent freezing was used to divide shocked rats into LR and HR as done previously in our laboratory (Chen et al. 2012). The duration of this test was 6 min comprised initially of background noise of 45-50 dB for 3 min followed by the novel auditory tone of 9 kHz at 75 dB for 3 min.

Table 1. Timeline of of the behavioral procedures in the modified model of PTSD.

Time	Behavioral procedure
Day 0	Footshock exposure
Day 1	Exposure to a novel tone in a novel chamber
Day 14	Perfusion and tissue preparation

The experiments were videotaped and subsequently the amount of time spent immobile (freezing) during the tone was scored for each rat by two observers who were blinded to group assignment. Based on the percentage of the total amount of time spent immobile (freezing duration /3 min × 100), shocked rats were assigned to LR (freezing % < 40 %) and HR (freezing % > 60 %). Rats that fell outside this range were discarded from the experiment. Previous work has shown that this 40/60 % split produces a group of shocked rats with similar level of anxiety to nonshocked rats (LR) and a group of shocked rats with high level of anxiety (HR) (Chen et al. 2012). After that, the rats were not exposed to any behavioral test for the 13 days prior to being sacrificed.

2.6.4. Tissue Preparation and RT-PCR

Fourteen days after the footshock exposure, the rats were anesthetized with chloral hydrate (600 mg/kg, i.p.) and perfused as described in section 2.5.2. The brains were quickly removed and the posterior halves of the hypothalamus were dissected on ice using a stereomicroscope. The mRNA level of ppOX was quantified with RT-PCR as described in section 2.5.3. The custom oligonucleotide primers for amplification of ppOX mRNA were obtained from Integrated DNA Technologies and the sequences were as follows: forward 5'-CGT CTT CTA TCC CTG TCC TAG T-3' ; and reverse, 5'-ACA CCA ACA GAG AAA CGT CTT TA-3'. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal quantitative control.

2.6.5. Statistical Analysis

StepOne™ software version 2.1 was used to obtain the RQ value of each sample and one-way ANOVA was used to compare the ppOX mRNA level in nonshock, LR, and HR, followed by LSD post hoc test to compare the group differences. Student's t-test was used for analysis of freezing between shocked and nonshocked rats.

2.7. Effects of Footshocks on Anxiety-Like Behaviors and mRNA Levels of Precursor Peptides for CRF and Opioids in the Forebrain

2.7.1. Footshock Procedure

Rats received an episode of unpredictable footshocks (5 × 2 s of 1.5 mA shocks presented randomly over 3 min with an intershock period of 10-50 s) as previously described in section 2.5.1. Nonshocked rats were placed in the chamber for the same amount of time but no shock was delivered. The timeline of this experiment is shown on Table 2.

2.7.2. Acute Fear Response to a Novel Tone

Shocked rats were grouped into LR and HR based on the amount of freezing duration in response to a novel tone (described in section 2.6.3) (Chen et al. 2012). Briefly, this was done by placing rats in a novel chamber where they were exposed to a novel tone (9 kHz, 75 dB against background noise levels of 45-50 dB). Based on the percentage of the total amount of time spent immobile, shock rats were assigned to LR (freezing % < 40 %) and HR (freezing % > 60 %).

Table 2. Timeline of the behavioral procedures for contextual fear and anxiety in the modified model of PTSD.

Time	Behavioral procedure
Day 0	Footshock exposure
Day 1	Exposure to a novel tone in a novel chamber
Day 2	Exposure to shock chamber
Day 10-11	Elevated T-maze Test
Day 14	Perfusion and tissue preparation

2.7.3. Fear Response to the Footshock Chamber

Fear to the shock context was assessed by measuring freezing to the shock chamber at 2 days after the footshock episode. The freezing behavior was measured to assess the fear response to the shock context. Rats were placed back in the footshock chamber for 5 min. All behaviors within this 5 min were recorded by a camera and subsequently the freezing duration of each rat to the context was scored by two observers who were blinded to group assignment. The percentage of freezing (freezing duration/5 min × 100) was counted as the fear level to the shock context. The footshock chamber was cleaned after each exposure of rats as in the footshock procedure described previously.

2.7.4. Elevated T-maze Test

At 10-11 days after the shock exposure, the elevated T-maze test was used to assess the anxiety level of subjects. It consisted of three arms (one closed arm and two open arms) which were elevated 50 cm from the floor. The closed arm (Plexiglass; length 50 cm × width 10 cm × height 30 cm) was perpendicular to the two open arms (Plexiglass; length 50 cm × width 10 cm × height 0.5 cm) and the whole elevated T-maze equipment was placed in a test room with dim light (3-5 lx). The rats were transferred to the test room and adapted for 1 hour on the day before the elevated T-maze test. This test measures the avoidance and escape behaviors of rats and includes a baseline trial, 2 avoidance trials and 2 escape trials. First of all, a rat was placed at the distal end of the closed arm facing the interaction zone (the

intersection of closed arm and open arms) and the latency for the rat going out of the closed arm with all four paws was measured (baseline trial). The trial was terminated if the rat went out of the closed arm or stayed in the closed arm for 5 min. Then the rat was placed back in the home cage for 30 s before the next trial. The same procedure as the baseline trial was repeated twice (avoidance 1 & 2 trials). After that, 2 escape trials were done by placing the rat at the distal end of the open arm facing the interaction zone and the latency for the rat going into the closed arm with all four paws was measured (escape 1 & 2 trials). If the rat went into the closed arm or stayed in the open arm for 5 min, the trial was terminated and the rat was placed back in the home cage. There was a 30 s interval between every two trials in which the test rat was in the home cage and the T-maze was cleaned with 0.5% liquinox. All trials were videotaped and the latency of each trial was measured for each rat by two observers who were blinded to group assignment.

2.7.5. Tissue Preparation and RT-PCR

At fourteen days post-shock, the rats were anaesthetized using chloral hydrate (600 mg/kg, i.p.) and perfused before removal of the brain as previously described in section 2.5.2. Samples for the nucleus accumbens (core and shell) as well as the medial and lateral caudate-putamen were dissected from sections at the level of the striatum (1.0 to 2.0 mm anterior to bregma; Fig.3A). Coronal sections were taken to dissect out the BST (0.0 to 1.0 posterior to bregma; Fig.3B) and the amygdala samples (1.8 to 2.8 posterior to bregma; Fig.3C). Amygdala samples contained all

of the CeA and relatively small portions of the basolateral and medial amygdala. Tissues were collected and stored at -80 °C for later analysis.

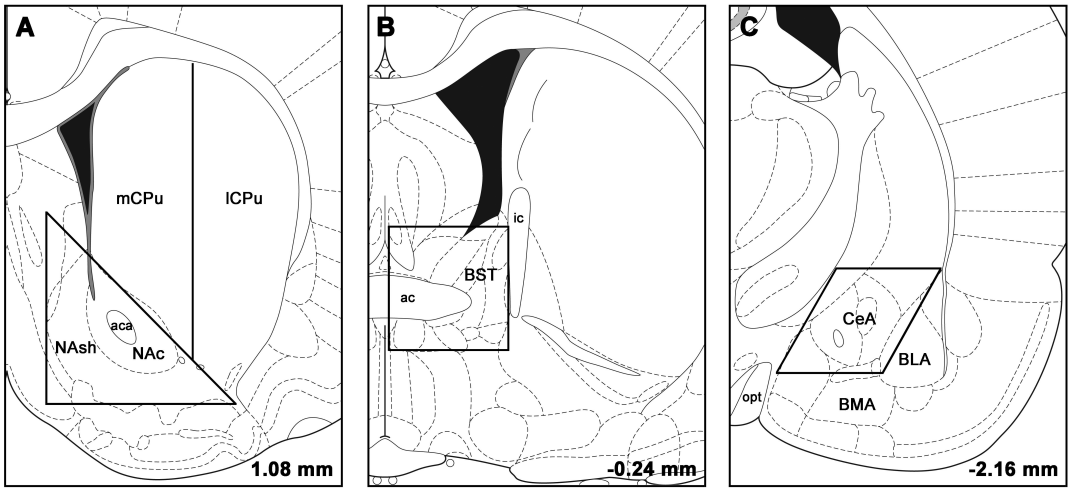
RT-PCR was performed to quantify the levels of the precursor genes for CRF, dynorphin, and enkephalin in subregions of the extended amygdala and striatum in shocked rats with anxiety-like behavior (HR) and no anxiety (LR) (Chen et al. 2012). The housekeeping gene Hprt1 was used as the internal quantitative control. The primers used were: Hprt-1 forward, 5'- CTC ATG GAC TGA TTA TGG ACA GGA C-3' and reverse, 5'-GCA GGT CAG CAA AGA ACT TAT AGC C-3'; proCRF forward, 5'-CAT CTC TCT GGA TCT CAC CT-3' and reverse, 5'-TTG CTG AGC TAA CTG CTC TG-3'; proDYN forward, 5'-TGT CCT TGT GTT CCC TGT G-3' and reverse, 5'-CAC TCC AGG GAG CAA ATC AG-3'; proENK forward, 5'-AAC TTC CTG GCA TGC ACA CT-3', and reverse, 5'-CTC ATC CTG TTT GCT GCT GTC-3'.

2.7.6. Statistical Analysis

A one-way ANOVA was used to evaluate the effects of different groups on the percentage of freezing to the novel tone and the shock chamber. Group differences in the elevated T-maze were assessed by Kruskal-Wallis one-way analysis of variance by ranks followed by a multiple comparison non-parametric adapted t-test when appropriate. Results from the RT-PCR experiments were analyzed using a one-way ANOVA with post hoc analysis with Dunnett's T3 tests if the overall ANOVA was significant. SPSS software (version 22.0) was used to perform all the data analyses.

Fig. 3. Diagrammatic representation of the regions of the brain dissected for analysis of propeptide mRNA (adapted from Paxinos and Watson, 2009).

Numbers on the bottom indicate the distance from bregma and represents approximately the center of the area sampled. Abbreviations (A) aca: anterior commissure, anterior part; lCPu: lateral caudate putamen; mCPu: medial caudate putamen; NAc: nucleus accumbens, core; NAsh: nucleus accumbens, shell; (B) ac: anterior commissure; BST: bed nucleus of the stria terminalis; ic: internal capsule; (C) BLA: basolateral amygdala; BMA: basomedial amygdala; CeA: central nucleus of amygdala; opt: optic tract.


A
mCPu
lCPu
aca
NAsh
NAc
1.08 mm
B
ic
BST
ac
-0.24 mm
C
CeA
BLA
opt
BMA
-2.16 mm

2.8. Changes of Contextual Fear and Anxiety Levels After Footshocks

2.8.1. Footshock Procedure

Rats received an episode of unpredictable footshocks (5 × 2 s of 1.5 mA shocks presented randomly over 3 min with an intershock period of 10-50 s) as previously described in section 2.5.1. At the same time, nonshocked rats were placed in the chamber for the same amount of time but no shock was delivered. The timeline of the present experiment is shown on Table 3.

2.8.2. Social Interaction Test

Shocked and nonshocked rats were tested for the anxiety in the social interaction test at 1 day post-shock (early time point), 2 weeks post-shock (middle time point) and 4 weeks post-shock (late time point), respectively.

The social interaction test is usually used to assess the level of social anxiety. A big chamber (Plexiglas; length 65 cm × width 40 cm × height 50 cm) was divided into 3 compartments: compartment A (length 15 cm × width 40 cm × height 50 cm), which was divided from the interaction zone with a metal mesh (40 cm × 50 cm); interaction zone (length 30 cm × width 40 cm × height 50 cm), which was the zone between the other two compartments; compartment B (length 20 cm × width 40 cm × height 50 cm), which was divided from the interaction zone with a sliding door (10 cm × 10 cm) in a black Plexiglas wall (40 cm × 50 cm) and the top of compartment B was covered to become a dark zone.

Table 3. Timeline of the behavioral tests for anxiety and contextual fear after footshocks.

Time	Behavioral procedure
Day 0	Footshock exposure
Day 1	Social Interaction Test (Early)
Day 2	Exposure to shock chamber (Early)
Day 14	Social Interaction Test (Middle)
Day 15	Exposure to shock chamber (Middle)
Day 28	Social Interaction Test (Late)
Day 29	Exposure to shock chamber (Late)

The social interaction test was done in a test room with dim light (3-5 lx) and rats adapted in the test room for 1 hour at the day before the test. This test was conducted as previous work in our laboratory (Chen et al. 2012). Briefly, a test rat was placed in the compartment B with the sliding door closed and another rat which was unfamiliar to the test rat was placed in the compartment A. Three minutes later, the sliding door was opened allowing the test rat to explore freely in the compartment B and interaction zone and interact with the unfamiliar rat through the metal mesh for 5 min. The experiment was videotaped after the sliding door was opened and subsequently the latency for the test rat going into the interaction zone was measured for each rat. The chamber was cleaned with 0.5% liquinox after each test.

2.8.3. Fear Response to the Footshock Chamber

Similarly, fear level of shocked and nonshocked rats was tested in the shocked chamber at 2 day post-shock (early time point), 2 weeks post-shock (middle time point) and 4 weeks post-shock (late time point), respectively. The freezing behavior was measured to assess the fear response to the shock context as previously described in section 2.7.3.

2.8.4. Statistical Analysis

A two-way ANOVA was used to evaluate the main and interaction effects of "shock" and "time". Pairwise comparisons were used to determine if differences

between groups were significant at each time point when appropriate.

2.9. Effects of Orexin Receptor Antagonists on Contextual Fear Conditioning and Anxiety Induced by Footshocks

2.9.1. Drugs and Injections

The dual orexin receptor antagonist TCS1102 (N-[1,1'-Biphenyl]-2-yl-1-[2-[(1-methyl-1H-benzimidazol-2-yl)thio]acetyl-2-pyrrolidinedicarboxamide; Tocris, Bristol, UK), OX1R antagonist SB334867 (N-(2-Methyl-6-benzoxazolyl)-N'-1,5-naphthyridin-4-yl urea; Tocris, Bristol, UK), and OX2R antagonist TCSOX229 ((2S)-1-(3,4-Dihydro-6,7-dimethoxy-2(1H)-isoquinolinyl)-3,3-dimethyl-2-[(4-pyridinylmethyl)amino]-1-butanone hydrochloride; Tocris, Bristol, UK) were purchased for this study. The dual orexin receptor antagonist TCS1102 was dissolved in a vehicle of PEG200 (poly-ethylene glycol 200; Sigma-Aldrich, Ontario, Canada). The OX1R antagonist SB334867 was dissolved in a vehicle of dimethyl sulfoxide (DMSO ; Amresco, Ohio, USA) whereas the OX2R antagonist TCSOX229 was dissolved in saline. All of the three drugs were administered by i.p. injection in a dose volume of 2 ml/kg 30 min before the behavioral tests.

Shocked and nonshocked rats were given either PEG200 or 20 mg/kg TCS1102 (i.p.) before being tested in the social interaction chamber. Another group of shocked rats received an injection of DMSO or one of two doses of OX1R antagonist

SB334867 (20 or 30 mg/kg; i.p.) whereas a third group was injected with saline or the OX2R antagonist TCSOX229 (20 or 30 mg/kg; i.p.).

2.9.2. Footshock Procedure

Rats received a single episode of footshocks (5 × 2 s of 1.5 mA) as previously described in section 2.5.1. At the same time, nonshocked rats were placed in the chamber for the same amount of time but no shock was delivered.

2.9.3. Social Interaction Test and Fear Response to the Footshock Chamber

At 2 weeks post-shock, the rats were placed in the social interaction chamber to test the level of anxiety 30 min after the injections of the orexin receptor antagonists as described in section 2.8.2. The latency for the test rat going into the interaction zone was measured for each rat as an index of anxiety. After the social interaction test, the rats were returned to their home cages and kept for 60 s and then placed in the shock chamber 5 min for assessment of contextual fear as described in section 2.7.3. Freezing to the shock context was scored for each rat and that the percentage of freezing (freezing duration/5 min × 100) was calculated. All of the results were analyzed by two experimenters blind to the condition. The correlation coefficient for two observers ranged from 0.92-0.97 and a mean of the two experimenters' score was used for data analysis.

2.9.4. Statistical Analysis

Group differences for the effects of dual orexin antagonist on the latency going into the interaction zone and the contextual freezing were assessed by the two-way ANOVA to evaluate the main and interaction effects of "shock" and "dual orexin receptor antagonist". A one-way ANOVA was used to assess group differences for the effects of the specific antagonists on the latency going into the interaction zone and the contextual freezing with post hoc Dunnett test when appropriate. The Statistical Package for the Social Sciences (SPSS; version 22.0) software was used to perform all the data analyses.

Chapter 3

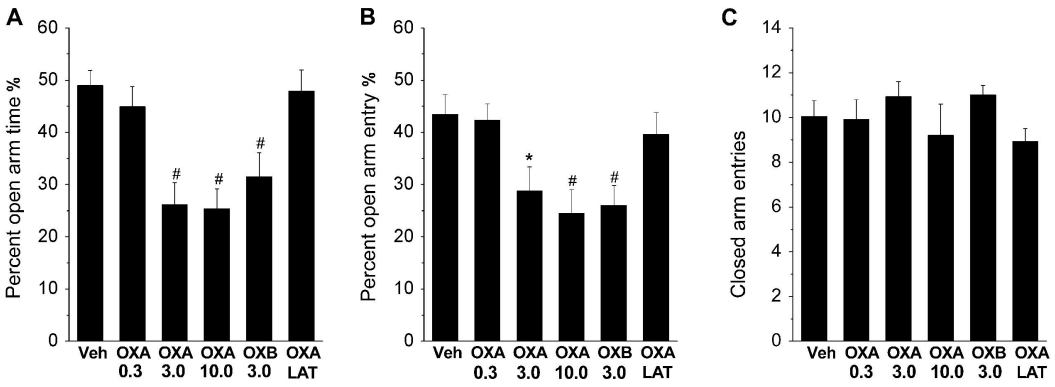
Results

3.1. Effects of Orexins Microinjections in the PVT on Anxiety Response

3.1.1. Spatiotemporal Measures of Anxiety in the Elevated Plus Maze

The effect of orexin-A microinjections in the PVT (0, 0.3, 3.0, and 10.0 μg, n = 10 to 11) or lateral thalamus (3.0 μg, n = 13) on the behavioral activity of rats placed in the elevated plus maze was assessed. The ANOVA revealed a significant main effect for treatment on the percent open arm time (Fig. 4A, $F_{(5,64)} = 7.177$; $p < 0.001$) and the percent open arm entry (Fig. 4B, $F_{(5,64)} = 4.682$, $p < 0.01$). Post hoc analysis showed that the percent open arm time ($p < 0.001$, $p < 0.001$) and the percent open arm entry ($p < 0.05$, $p < 0.01$) were significantly decreased in rats treated with the higher concentrations of orexin-A (3.0 and 10.0 μg) when compared to the vehicle treated group (Fig. 4A and 4B). There were no significant differences between rats received the low concentration of orexin-A (0.3 μg) and saline ($p > 0.05$). Since orexin-A binds with equal strength to both the OX1R and the OX2R, whereas orexin-B binds preferentially to the OX2R, we examined the effect of stimulating OX2R by microinjecting orexin-B (3.0 μg, n = 14) in the PVT. Similar to the higher concentrations of orexin-A, administrations of orexin-B (3.0 μg) in the PVT resulted in a reduction in the percent open arm time ($p < 0.01$; Fig. 4A) and the percent open arm entry ($p < 0.01$; Fig. 4B). These results indicate that the orexin-A and orexin-B

Fig. 4. Effects of orexin-A (OXA) or orexin-B (OXB) microinjections in the paraventricular nucleus of the thalamus (PVT) region and lateral thalamus (LAT) on anxiety assessed by spatiotemporal measurement in the elevated plus maze. Effects of OXA (0.3, 3.0 and 10.0 μg), OXB (3.0 μg), or vehicle (Veh) in the PVT region and LAT on percent open arm time (A), percent open arm entry (B), and closed arm entries (C). The values represent mean ± SEM for this and all subsequent figures. *indicates $p<0.05$ and $^{\#}p<0.01$ compared to vehicle, n=10 to14 for each group.


A
Percent open arm time %
60
50
40
30
20
10
0
#
#
#
Veh
OXA 0.3
OXA 3.0
OXA 10.0
OXB 3.0
OXA LAT
B
Percent open arm entry %
60
50
40
30
20
10
0
*
#
#
Veh
OXA 0.3
OXA 3.0
OXA 10.0
OXB 3.0
OXA LAT
C
Closed arm entries
14
12
10
8
6
4
2
0
Veh
OXA 0.3
OXA 3.0
OXA 10.0
OXB 3.0
OXA LAT

microinjections in the PVT region produce anxiogenic effects in the elevated plus maze. The behavioral effect of microinjections of orexin-A (3.0 μg) into the lateral thalamus was measured and compared with microinjections of orexin-A or vehicle in the midline thalamus. The post hoc comparison revealed that orexin-A in the lateral thalamus had no effect on the percent open arm time and percent open arm entry compared to the vehicle group (Fig. 4A and 4B). The post hoc analysis also indicated that orexin-A in the PVT decreased the percent open arm time and percent open arm entry compared to microinjections of orexin-A in the lateral thalamus ($p < 0.01$, $p < 0.05$; Fig. 4A and 4B). As such, the results indicate that the behavioral effects of microinjections of orexin-A and orexin-B in the midline thalamus were due to stimulation of orexin receptors in the PVT.

There were no significant differences between any of the treatments on the number of entries in the closed arm (Fig. 4C, $F_{(5,64)} = 1.714$, $p > 0.05$), a measure of locomotor activity in the elevated plus maze (Cruz, Frei, and Graeff 1994, Espejo 1997, File 2001). This indicates that the anxiety-like effects produced by microinjections of orexin-A and orexin-B into the PVT were not caused by impairment of locomotion.

3.1.2. Ethological Measures of Anxiety in the Elevated Plus Maze

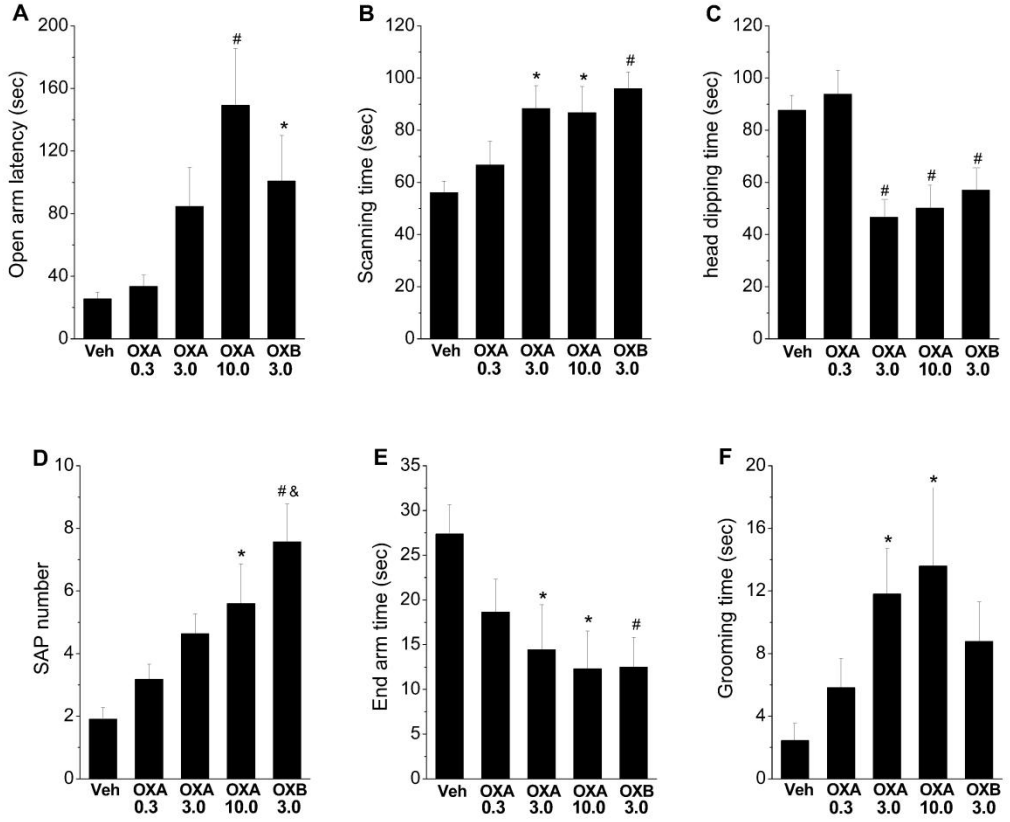
Increases in open arm latency and scanning time are measures of hesitation in an approach-avoidance conflict, as well as the avoidance of unprotected areas in the elevated plus maze (Espejo 1997, Ohl et al. 2001, Wall and Messier 2001). The

ANOVA showed significant main treatment effects for open arm latency (Fig. 5A, $F_{(5,64)} = 4.197$, $p < 0.01$) and scanning time (Fig. 5B, $F_{(5,64)} = 4.751$, $p < 0.01$). Post hoc analysis indicated that orexin-A (10.0 μg, $p < 0.01$) and orexin-B (3.0 μg, $p < 0.05$) in the PVT significantly increased the open arm latency compared to the vehicle group (Fig. 5A). In addition, orexin-A (3.0 and 10.0 μg, $p < 0.05$, $p < 0.05$) and orexin-B (3.0 μg, $p < 0.01$) increased the scanning duration compared to the vehicle group (Fig. 5B).

An increase in the number of SAP is hypothesized to be a measure of risk assessment in rodents tested in the elevated plus maze (Augustsson and Meyerson 2004, Rodgers and Johnson 1995). There was a significant difference among the groups for the number of SAP (Fig. 5D, $F_{(5,64)} = 6.47$, $p < 0.001$) and post hoc comparisons revealed that rats receiving the orexin-A (3.0 μg, $p < 0.05$; 10.0 μg, $p < 0.01$) or orexin-B (3.0 μg, $p < 0.001$) displayed more SAP compared to vehicle treated rats (Fig. 5D). In addition, the rats treated with orexin-B (3.0 μg, $p < 0.05$) showed more SAP compared to the ones treated with the same concentration of orexin-A (Fig. 5D).

A decrease in the end arm time and head dipping time indicates a decrease in active exploration of uncertain areas in the elevated plus maze (Borelli and Brandao 2008, Silva and Frussa-Filho 2000, Wall and Messier 2001). In addition, an increase in displacement behaviors such as grooming is usually associated with a stressful or conflict situation and represents behavioral correlates of a negative emotional state (Berridge et al. 1999, Espejo 1997). The ANOVA showed significant differences

Fig. 5. Effects of orexin-A (OXA) or orexin-B (OXB) microinjections in the paraventricular nucleus of the thalamus (PVT) on anxiety evaluated by the ethological measurement in the elevated plus maze. Effects of OXA (0.3, 3.0 and 10.0 μg), OXB (3.0 μg), or vehicle (Veh) in the region of the PVT on open arm latency (A), scanning time (B), head dipping time (C), number of stretch-attend postures (D), end arm time (E), and grooming time (F). *indicates $p < 0.05$ and #indicates $p < 0.01$ compared to Veh, &indicates $p < 0.05$ compared to OXA (3.0 μg), n = 10 to 14 for each group.


A
Open arm latency (sec)
200
160
120
80
40
0
#
*
B
Scanning time (sec)
120
100
80
60
40
20
0
*
*
#
C
head dipping time (sec)
120
100
80
60
40
20
0
#
#
#
Veh
OXA 0.3
OXA 3.0
OXA 10.0
OXB 3.0
D
SAP number
10
8
6
4
2
0
*
#&
E
End arm time (sec)
35
30
25
20
15
10
5
0
*
*
#
F
Grooming time (sec)
20
16
12
8
4
0
*
*

for the head dipping time (Fig. 5C, $F_{(5,64)} = 7.613$, $p < 0.001$), end arm time (Fig. 5E, $F_{(5,64)} = 2.482$, $p < 0.05$), and grooming time (Fig. 5F, $F_{(5,64)} = 2.21$, $p < 0.05$) among treatment groups. Post hoc comparison showed that rats injected with orexin-A (3.0 and 10.0 μg) and orexin-B (3.0 μg) showed significant decreases in head dipping time ($p < 0.01$, $p < 0.01$, $p < 0.05$; Fig. 5C) and end arm time ($p < 0.05$, $p< 0.05$, $p < 0.05$; Fig. 5E). Only orexin-A treated rats (3.0 and 10.0 μg, $p < 0.05$, $p < 0.05$) showed increased grooming time compared to the vehicle group, whereas orexin-B (3.0 μg) treated rats did not show that increase ($p = 0.15$; Fig. 5F). No differences were found for the number of defecation episodes between the different treatment groups (data not shown, $F_{(5,64)} = 0.495$, $p > 0.05$). Finally, there were no differences in the expression of ethological behaviors in rats receiving orexin-A in the lateral thalamus and rats receiving the vehicle in the PVT region.

3.2. Effects of CRF and Kappa Opioid Receptor Blockade on Anxiety Produced by Orexin-A Microinjection in the PVT

3.2.1. Spatiotemporal Measures of Anxiety in the Elevated Plus Maze

The CRF antagonist hCRF (1.0 μg, n = 9) or the kappa antagonist nBNI (10.0 μg, n = 9) was infused in the lateral ventricles before injecting orexin-A (3.0 μg) in the PVT. The ANOVA showed significant main effects for treatment on the percent open arm time (Fig. 6A, $F_{(3,34)} = 4.69$, $p < 0.01$) and the percent open arm entry (Fig. 6B, $F_{(3,34)} = 4.231$, $p < 0.01$) but not the number of closed arm entries ($F_{(3,34)} = 1.352$, $p > 0.05$, data not shown). Post hoc analysis showed that both

hCRF and nBNI significantly reversed the decrease in the percent open arm time ($p < 0.01$) and the percent open arm entry ($p < 0.01$) by orexin-A injection. In contrast, hCRF (n=12) and nBNI (n= 11) alone when compared with saline (n = 12) did not have any effect on the percent open arm time ($F_{(2,31)} = 0.05$, $p > 0.05$) or percent open arm entry ($F_{(2,31)} = 0.44$, $p > 0.05$) in rats that had received only saline in the PVT region.

3.2.2. Ethological Measures of Anxiety in the Elevated Plus Maze

The ethological analysis of behaviors in the elevated plus maze test was used to further examine the behavioral effect of both antagonists on the anxiety induced by orexin-A injection in the PVT. The ANOVA revealed significant main effects of treatment for the open arm latency (Fig. 7A, $F_{(3,34)} = 4.164$, $p < 0.05$), scanning time (Fig. 7B, $F_{(3,34)} = 7.022$, $p < 0.01$), head dipping time (Fig. 7C, $F_{(3,34)} = 7.421$, $p < 0.01$), SAP number (Fig. 7D, $F_{(3,34)} = 7.256$, $p < 0.01$), and grooming time (Fig. 7F, $F_{(3,34)} = 4.312$, $p < 0.01$), but no difference was observed on the end arm time (Fig. 7E, $F_{(3,34)} = 1.422$, $p > 0.05$). Post hoc comparison demonstrated that nBNI significantly reversed the changes in open arm latency ($p < 0.05$), scanning time ($p < 0.01$), head dipping time ($p < 0.01$), and SAP number ($p < 0.05$) produced by orexin-A. Similarly, hCRF had similar effects on the open arm latency ($p < 0.05$), scanning time ($p < 0.01$), head dipping time ($p < 0.01$), SAP number ($p <0.01$), and grooming time ($p < 0.05$). No significant difference was observed on open arm latency ($F_{(2,31)} = 0.789$, $p > 0.05$), scanning time ($F_{(2,31)} = 0.331$, $p > 0.05$), head

Fig. 6. Effects of injections of norbinaltorphimine (nBNI) and α-helical corticotropin releasing factor (hCRF) following microinjections of orexin-A (OXA) in the paraventricular nucleus of the thalamus (PVT) in the elevated plus maze. Effects of intracerebroventricular injections of nBNI (10.0 μg), hCRF (1.0 μg) or vehicle (Veh) on the percent open arm time (A) and percent open arm entry (B) following microinjections of OXA (3.0 μg) in the PVT. *indicates $p < 0.05$ compared to Veh-OXA, n = 9 for each group.

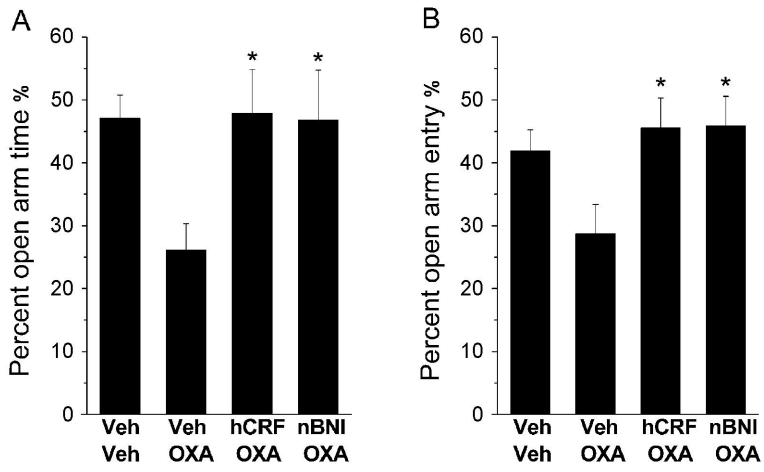
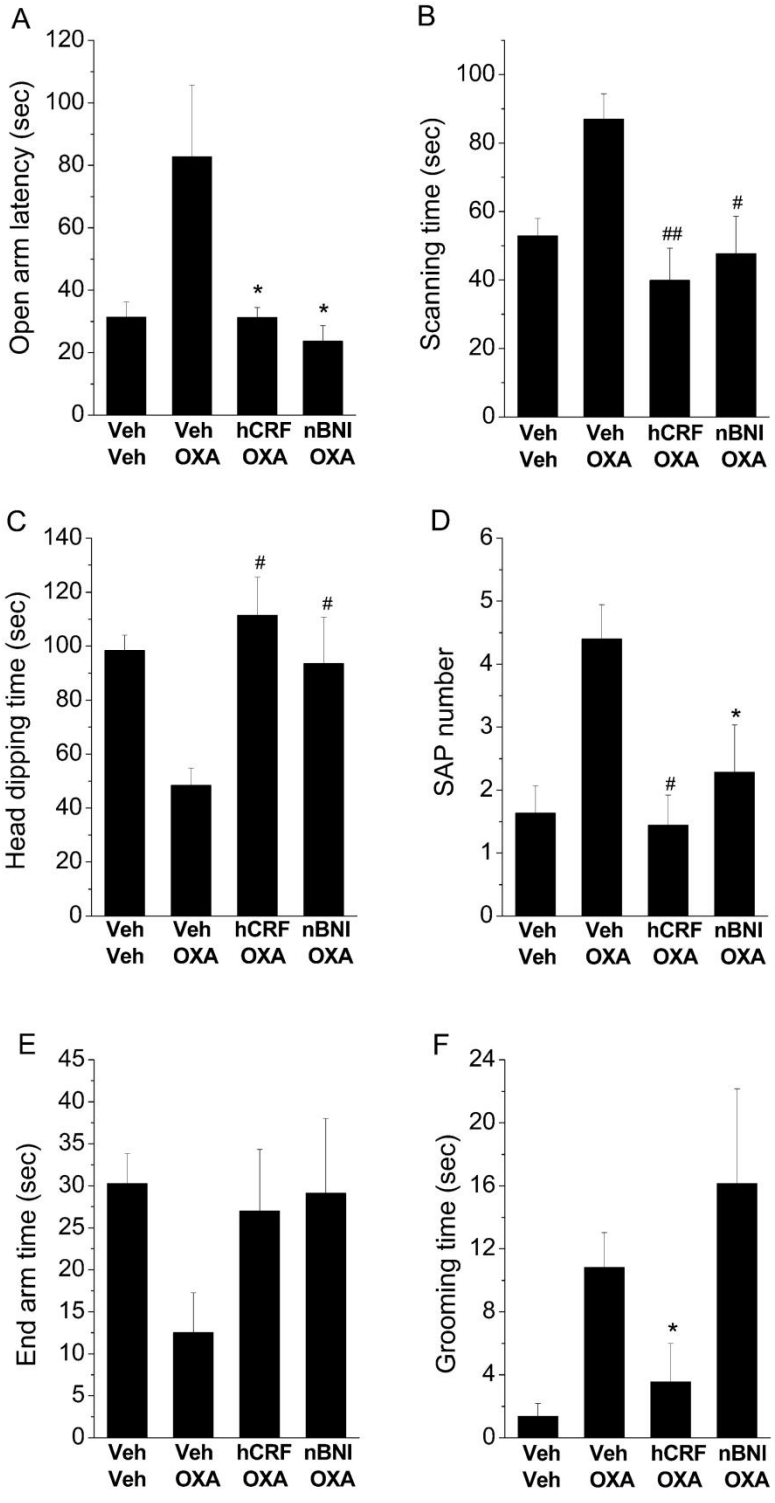

A
Percent open arm time %
60
50
40
30
20
10
0
*
*
Veh
Veh
Veh
OXA
hCRF
OXA
nBNI
OXA
B
Percent open arm entry %
60
50
40
30
20
10
0
*
*
Veh
Veh
Veh
OXA
hCRF
OXA
nBNI
OXA

Fig. 7. Effects of intracerebroventricular injections of norbinaltorphimine (nBNI) and α-helical corticotropin-releasing factor CRF (hCRF) following microinjections of orexin-A (OXA) in the PVT on anxiety evaluated by the ethological measurement in the elevated plus maze. Effects of intracerebroventricular injections of nBNI, hCRF, and vehicle (Veh) on changes in open arm latency (A), scanning time (B), head dipping time (C), SAP number (D), end arm time (E), and grooming time (F) produced by microinjections of 3.0 μg of OXA in the PVT region. *indicates $p < 0.05$ and #indicates $p < 0.01$ compared to Veh-OXA, n = 9 for each group.


A
Open arm latency (sec)
120
100
80
60
40
20
0
*
*
Veh Veh
Veh OXA
hCRF OXA
nBNI OXA
B
Scanning time (sec)
100
80
60
40
20
0
##
#
Veh Veh
Veh OXA
hCRF OXA
nBNI OXA
C
Head dipping time (sec)
140
120
100
80
60
40
20
0
#
#
Veh Veh
Veh OXA
hCRF OXA
nBNI OXA
D
SAP number
6
5
4
3
2
1
0
#
*
Veh Veh
Veh OXA
hCRF OXA
nBNI OXA
E
End arm time (sec)
45
40
35
30
25
20
15
10
5
0
Veh Veh
Veh OXA
hCRF OXA
nBNI OXA
F
Grooming time (sec)
24
20
16
12
8
4
0
*
Veh Veh
Veh OXA
hCRF OXA
nBNI OXA

dipping time ($F_{(2,31)} = 0.532$, $p > 0.05$), SAP numbers ($F_{(2,31)} = 0.399$, $p > 0.05$), end arm time ($F_{(2,31)} = 0.875$, $p > 0.05$) and grooming time ($F_{(2,31)} = 0.178$, $p > 0.05$) when infusing hCRF or nBNI into lateral ventricle before injecting saline into the midline thalamus.

3.3. Long-Lasting Changes of the Orexin System in Rats Exposed to Footshocks

The mRNA levels for ppOX ($t_{20} = 2.51$, $p < 0.05$; Fig. 8A) and the OX1R ($t_{21} = 2.16$, $p < 0.05$; Fig. 8B) were significantly higher in the posterior hypothalamus of shocked rats. In contrast, the mRNA level for the OX2R was not different in the hypothalamus between shocked and nonshocked rats (Fig. 8C). In addition, an increase in the expression of the OX1R in the posterior hypothalamus of shocked rats was confirmed using western blot analysis. In these experiments, OX2R antibody produced a single band near the 51 kDa associated with this receptor whereas the OX1R antibody produced a band near the expected 48 kDa molecular weight for this receptor. The level of the OX1R protein levels was significantly increased in shocked rats ($t_{20} = 3.02$, $p < 0.01$; Fig. 8D) compared to nonshocked rats. In contrast, differences in OX2R protein levels did not reach significant levels between shocked and nonshocked rats (Fig. 8E). The mRNA levels for the OX1R and the OX2R were not different in the midline thalamus (Fig. 9A) or the locus coeruleus/parabrachial area (Fig. 9B) between shocked and nonshocked rats.

Fig. 8. Levels of mRNA for prepro-orexin (ppOX) and orexin receptors and protein levels for orexin receptors in the hypothalamus. Levels of mRNA for the ppOX (A) and orexin 1 receptor (OX1R; B) were increased in the hypothalamus of shocked rats (n = 14 to15) compare to nonshocked rats (n = 8 to 9). The protein level for OX1R (D) was also increased in the shocked rats. The levels of orexin 2 receptor (OX2R; C & E) were not significantly different between shocked and nonshocked rats. The values are expressed as mean ± SEM. *$p < 0.05$, **$p < 0.01$ compared to the nonshocked group.

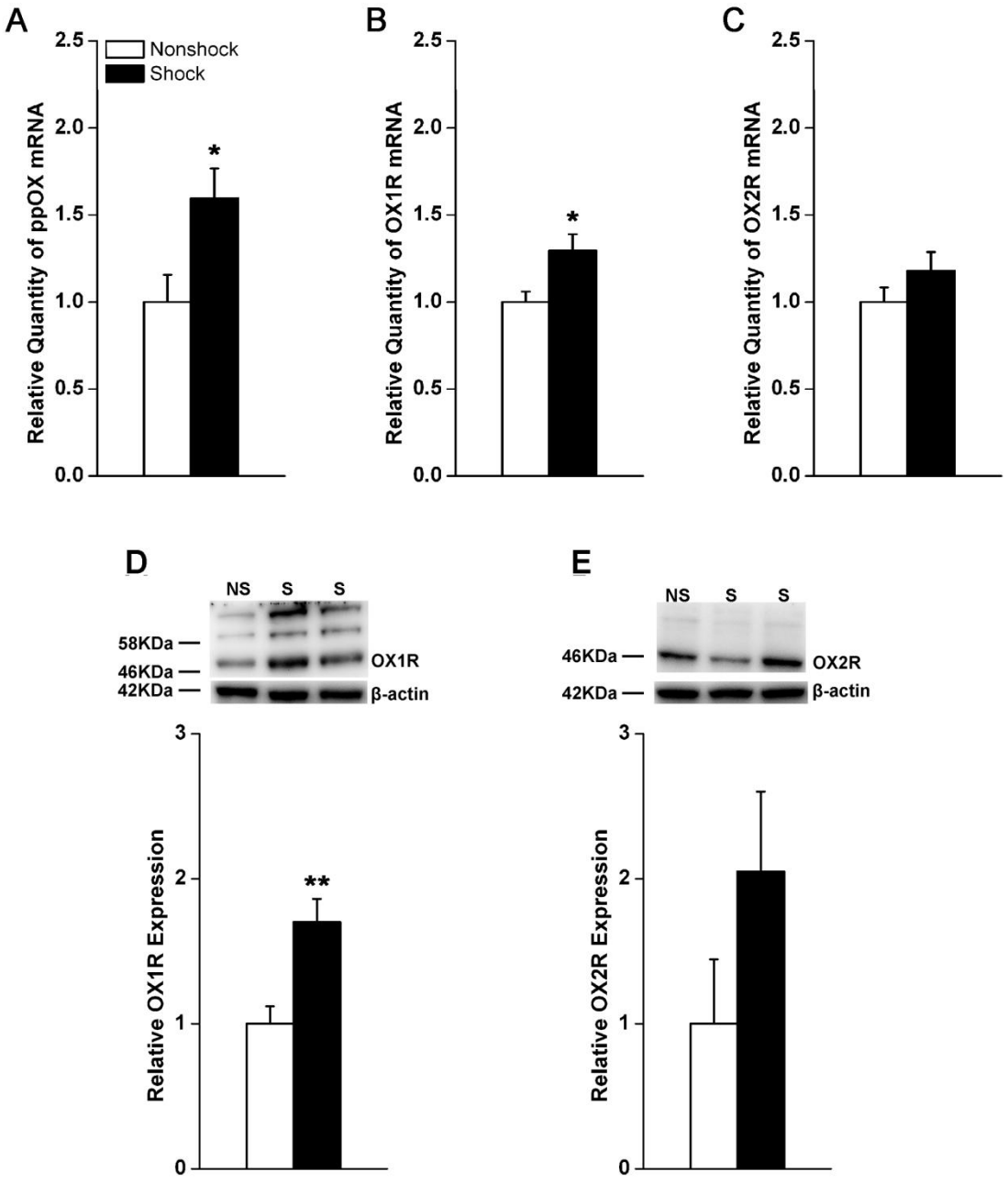
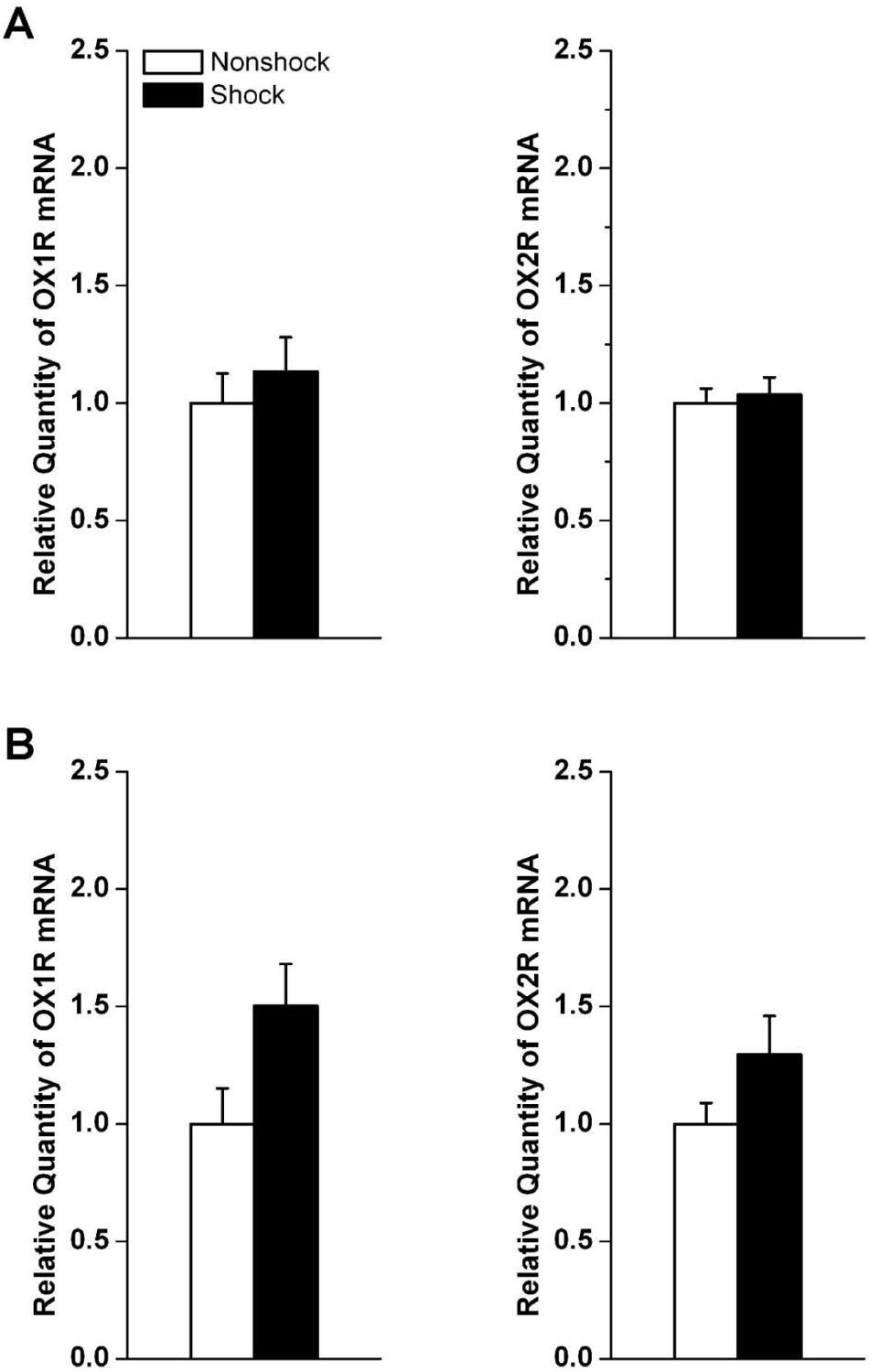

A
B
C
Nonshock
Shock
Relative Quantity of ppOX mRNA
Relative Quantity of OX1R mRNA
Relative Quantity of OX2R mRNA
2.5
2.0
1.5
1.0
0.5
0.0
*
D
E
NS
S
S
58KDa
46KDa
42KDa
OX1R
β-actin
OX2R
Relative OX1R Expression
Relative OX2R Expression
3
2
1
0
**

Fig. 9. Levels of mRNA for orexin 1 receptor (OX1R) and orexin 2 receptor (OX2R) in the midline thalamus and the locus coeruleus/parabrachial area.

The mRNA levels for OX1R and OX2R in the midline thalamus (A) and the locus coeruleus/parabrachial area (B) were not significantly different between shocked (n = 14 to15) compare to nonshocked rats (n = 8 to 9). The values in the histograms are mean ± SEM.


A
Nonshock
Shock
Relative Quantity of OX1R mRNA
Relative Quantity of OX2R mRNA
2.5
2.0
1.5
1.0
0.5
0.0
B
Relative Quantity of OX1R mRNA
Relative Quantity of OX2R mRNA
2.5
2.0
1.5
1.0
0.5
0.0

3.4. Long-Lasting Changes of the ppOX mRNA Level in a Modified Footshock Model of PTSD

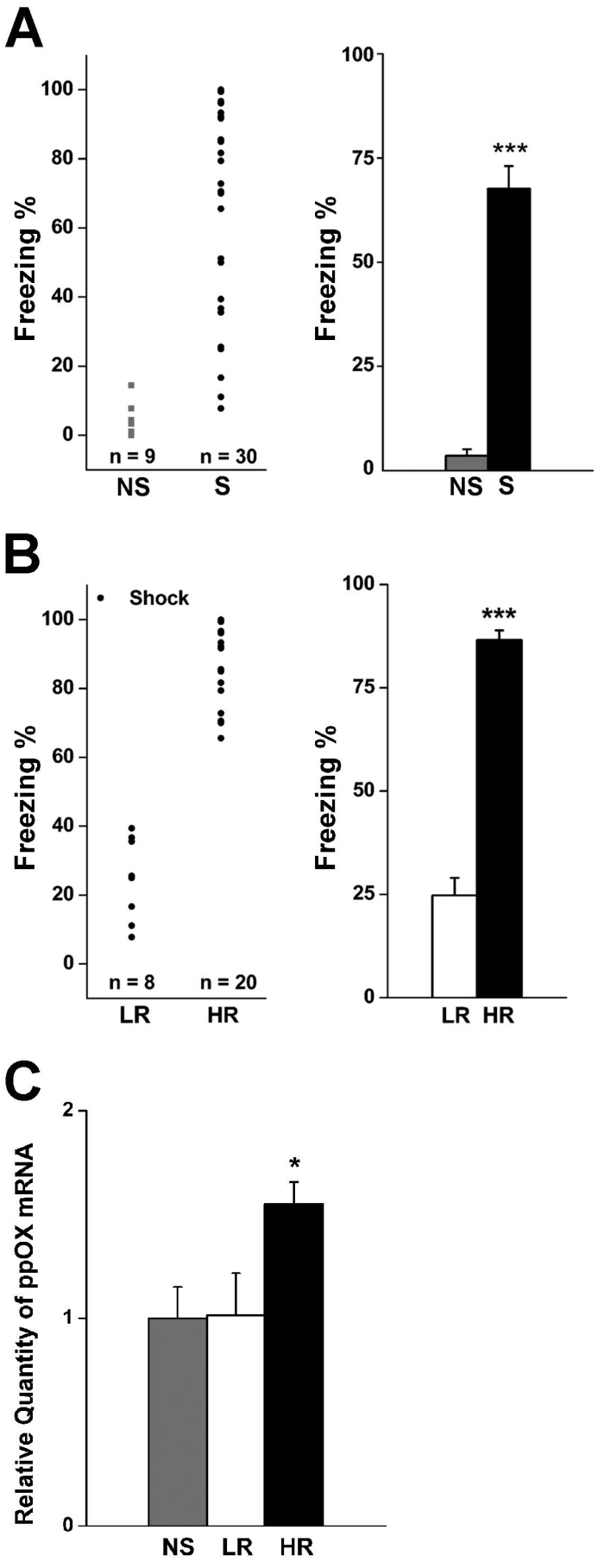
Shocked rats showed increased freezing ($t_{34} = 4.97$, $p < 0.001$) to the tone presented in a novel chamber as compared to the controls (Fig. 10A). The amount of freezing in shocked rats ranged from 0 to 100 %. Shocked rats that had freezing percentage scores < 40 % were selected for the LR group (n = 9) and those with a freezing percentage score > 60 % were selected to form the HR group (n = 16) with the remaining (freezing percentage ranging 40-60 %; n = 3) rats omitted from further analysis (Fig. 10B). The level of ppOX mRNA, which was measured with RT-PCR at 14 days after shock exposure, was found to be different between different groups ($F_{(2,30)} = 3.35$, $p < 0.05$; Fig. 10C) and the post hoc test indicated that HR had increased ppOX mRNA.

3.5. Effects of Footshocks on Anxiety-Like Behaviors and mRNA Levels of Precursor Peptides for CRF and Opioids in the Forebrain

3.5.1. Acute Fear Response to a Novel Tone

Shocked rats showed increased freezing to the novel tone ($F_{(1,38)} = 38.085$, $p < 0.001$) compared to the nonshocked rats when tested 24 hours after the footshock exposure (Fig. 11A). The percentage of time that shocked rats spent immobile ranged from 4.4 % to 100 %. Shocked rats that were freezing < 40 % were selected for the LR group (n = 9) and those that were freezing > 60% were selected for the HR group (n = 20) with the remaining rat omitted from further analysis (n = 1; Fig. 11B).

Fig. 10. Prepro-orexin (ppOX) mRNA level in shocked rats subgrouped into low responders (LR) and high responders (HR) at 14 days post-shock. (A) The individual freezing score and the mean freezing score for nonshocked (NS) and shocked rats (S) exposed to a novel tone 1 day after the shock exposure. (B) Individual freezing score and the mean freezing score for shocked rats assigned to the LR and HR groups. (C) The HR (n = 16) group shows an increase level of ppOX compared to the nonshocked (n = 8) and LR (n = 9) groups. The values in the histograms are mean ± SEM. *$p < 0.05$, ***$p < 0.001$ compared to nonshocked group (A & C), ***$p < 0.001$ compared to LR group (B).


A
Freezing %
100
80
60
40
20
0
n = 9
n = 30
NS
S
100
75
50
25
0
***
NS S
B
Shock
Freezing %
100
80
60
40
20
0
n = 8
n = 20
LR
HR
100
75
50
25
0
***
LR HR
C
Relative Quantity of ppOX mRNA
2
1
0
*
NS
LR
HR

Accordingly, there was a clear group difference in freezing between HR and LR ($F_{(1,27)}$ = 140.252, $p < 0.001$; Fig. 11B). Another group of nonshocked rats (n = 10) were used as controls.

3.5.2. Fear Response to Shock Chamber and Anxiety Response in the Elevated T-maze

One subject from the LR group was removed from the data set because its behavior in the elevated T-maze test was 2 standard deviations from the baseline latency mean for that group in that test. As such, the number of subjects for the behavioral experiments was as follows (10 nonshocked, 8 LR, and 20 HR). The amount of contextual fear measured 2 days after the footshock exposure was significantly different between nonshocked, LR and HR ($F_{(2,36)} = 91.128$, $p < 0.001$; Fig. 12A). The Dunnett's T3 post hoc test indicated that nonshocked group showed less freezing than LR and HR ($p < 0.001$).

The elevated T-maze test was used to test the avoidance tendencies of rats at 10-11 days post-shock. The Kruskal-Wallis test showed that there was a statistically significant difference on avoidance latency between different groups in baseline trial ($H = 13.430$, $p < 0.001$; Fig. 12B) and avoidance 1 trial ($H = 8.766$, $p < 0.05$; Fig. 12B). The comparison between different groups indicated that the HR displayed longer latency than the LR ($p < 0.05$) and nonshocked rats ($p < 0.001$) in the baseline trial. In contrast, LR and HR showed similar avoidance but only HR showed a significantly different latency compared to nonshocked rats ($p < 0.05$) in the

Fig. 11. Grouping shocked rats into low responders (LR) and high responders (HR). (A) The individual freezing score and the mean freezing score for nonshocked (NS) and shocked (S) rats exposed to a novel tone 24 hours after the footshock exposure. (B) Individual freezing score and the mean freezing score for shocked rats assigned to LR and HR groups. The values are expressed as mean ± SEM. ***$p < 0.001$ compared to nonshocked group (A), ***$p < 0.001$ compared to LR group (B).

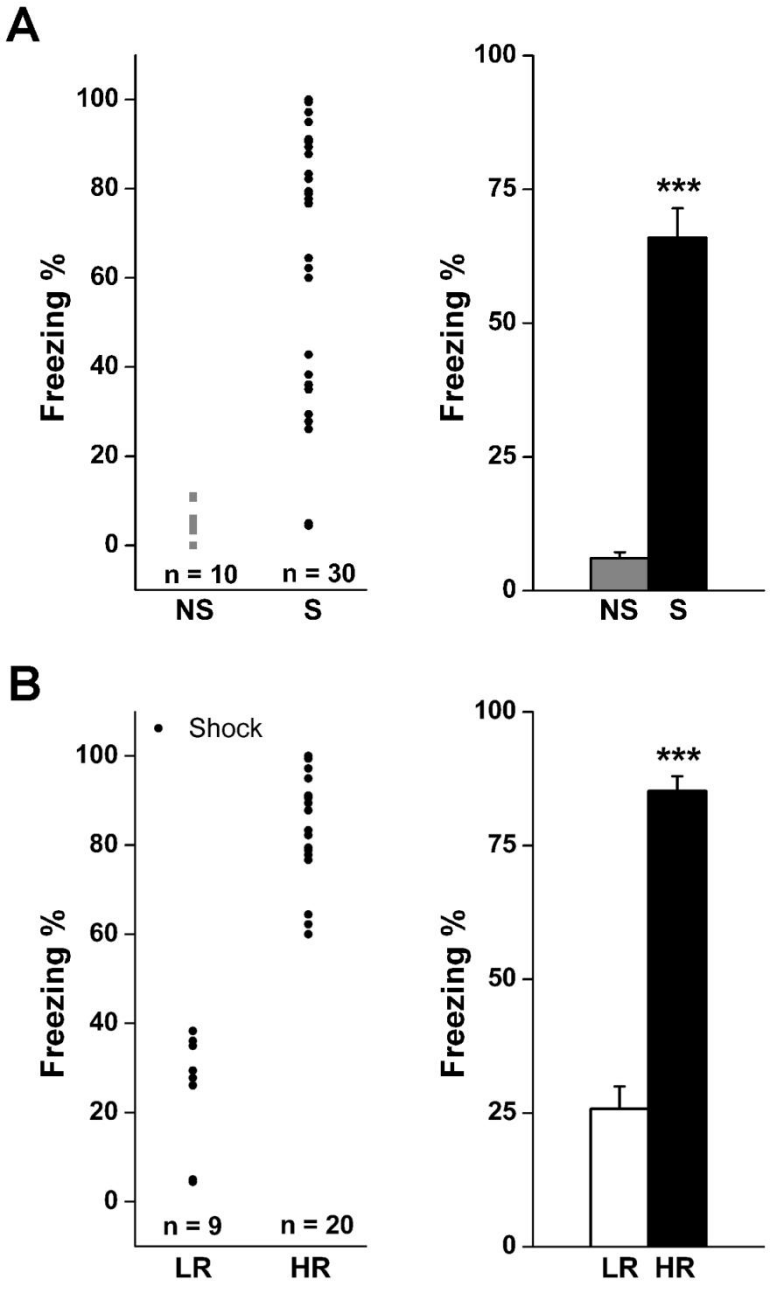
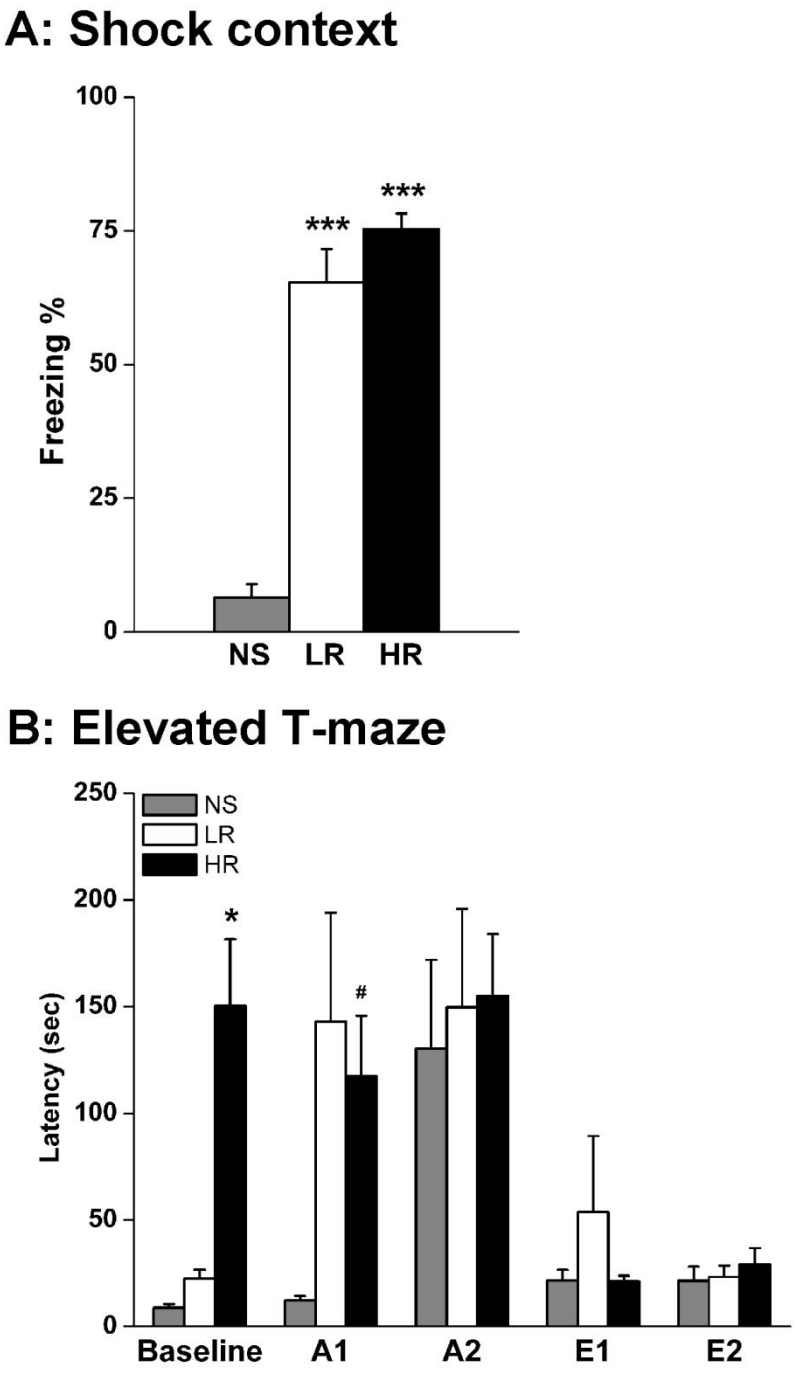

A
Freezing %
100
80
60
40
20
0
n = 10
n = 30
NS
S
Freezing %
100
75
50
25
0
***
NS
S
B
Shock
Freezing %
100
80
60
40
20
0
n = 9
n = 20
LR
HR
Freezing %
100
75
50
25
0
***
LR
HR

Fig. 12. Fear response to shock chamber and anxiety response in the elevated T-maze. (A) Low responders (LR) and high responders (HR) show increased freezing compared to nonshocked rats (NS). (B) HR display increased avoidance of the open arm in the baseline trial compared to LR and NS. The values in the histograms are mean ± SEM. ***$p < 0.001$ compared to nonshocked group (A); *$p < 0.05$ compared to the nonshocked and LR groups; #$p < 0.05$ compared to the nonshocked group (B). Abbreviations: A1, avoidance 1 trial; A2, avoidance 2 trial; E1, escape 1 trial; E2, escape 2 trial.


A: Shock context
100
75
50
25
0
Freezing %
***
***
NS
LR
HR
B: Elevated T-maze
250
200
150
100
50
0
Latency (sec)
NS
LR
HR
*
#
Baseline
A1
A2
E1
E2

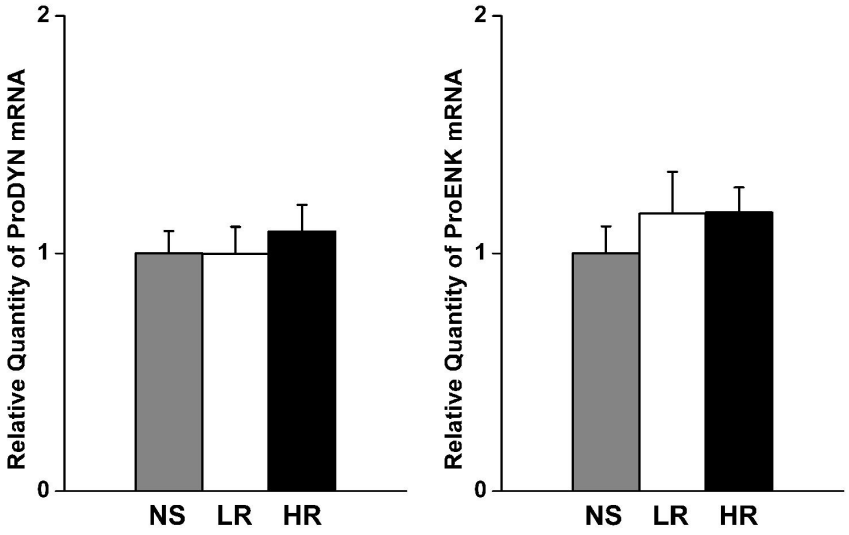
avoidance 1 trial. These data indicate that HR showed higher levels of anxiety-like behavior despite the fact that context fear was similar between LR and HR.

3.5.3. The mRNA Levels of proCRF, proDYN and proENK

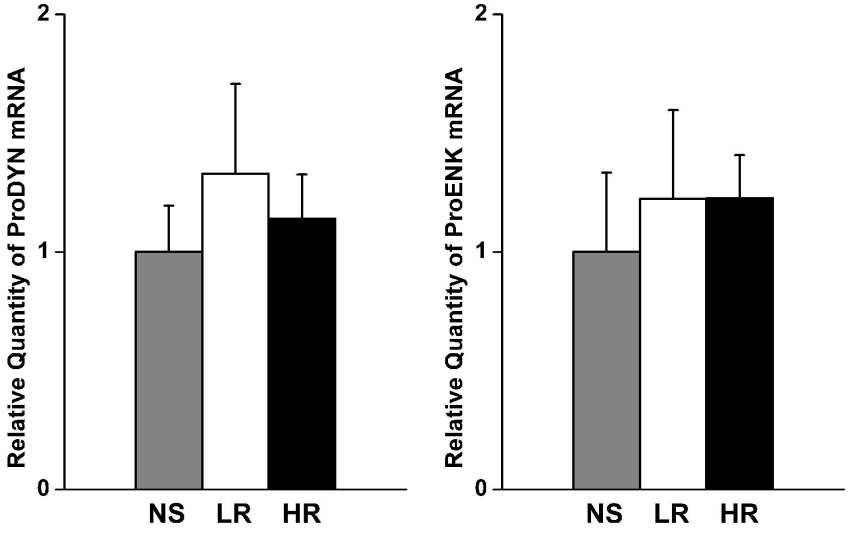
Some samples were lost during the processing phase but the total number of samples per brain regions analyzed was similar for all the RT-PCR experiment (n = 9 to 10 for nonshocked; n = 7 to 8 for LR; and n = 19 to 20 for HR). Neurons producing enkephalin and dynorphin are found throughout the striatum, the BST and the amygdala (Cassell, Freedman, and Shi 1999, Cassell, Gray, and Kiss 1986, Furuta, Zhou, and Kaneko 2002, Poulin et al. 2009). The present experiments examined the levels of the mRNA for proDYN and proENK in the extended amygdala and striatum of nonshocked, LR, and HR at 14 days post-shock (Fig. 13 and 14). The results showed that for all the brain regions examined, the mRNA levels for proDYN and proENK were not different between groups at 14 days after the shock exposure. Indeed, the levels of the mRNA for these two opioid peptides were very similar in HR, LR and nonshocked rats in the striatum (Fig. 13) and the extended amygdala (Fig. 14). Neurons that produce CRF are located in the amygdala, BST and caudal portion of the shell of the nucleus accumbens (Becker et al. 2007, Swanson et al. 1983). ProCRF mRNA levels for the BST and amygdala were quantified in the samples dissected from the brains of nonshocked, LR, and HR. The CRF neuronal population in the caudal nucleus accumbens was not assessed because of the difficulty in isolating this population of CRF neurons from those located anteriorly in the nucleus accumbens

Fig. 13. The mRNA levels of proDYN and proENK in the nucleus accumbens and caudate putamen. The levels of proDYN and proENK mRNA were not different between nonshocked (NS), low responders (LR) and high responders (HR) in the nucleus accumbens (A), lateral caudate putamen (B) and medial caudate putamen (C). The values are expressed as mean ± SEM.

A: Nucleus accumbens



B: Lateral caudate putamen



C: Medial caudate putamen

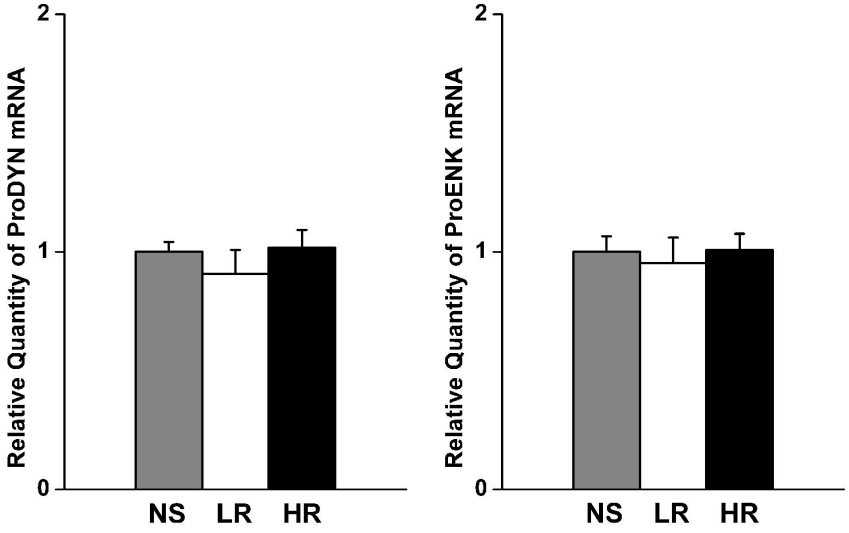
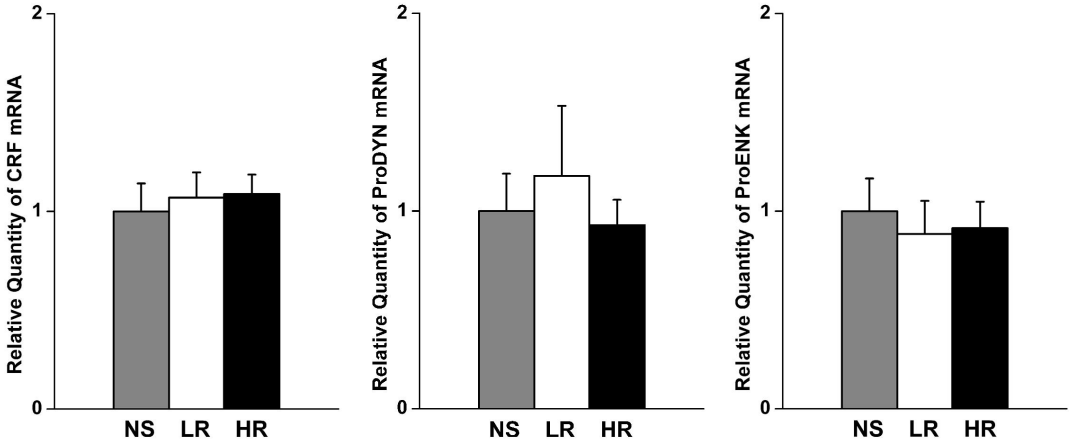
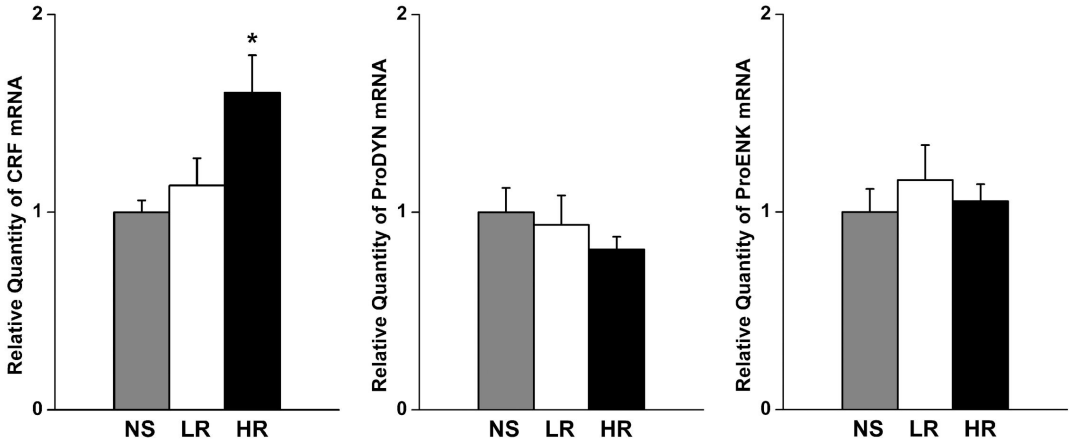


Fig. 14. The mRNA levels of proCRF, proDYN and proENK in the bed nucleus of the stria terminalis (BST) and amygdala. The levels of proDYN, proENK, and proCRF mRNA were similar in nonshocked (NS), low responders (LR) and high responders (HR) in the BST (A). In contrast, proCRF mRNA levels were increased in the HR compared to NS in the amygdala while the proDYN and proENK mRNA levels were similar across all groups (B). The values are expressed as mean ± SEM. *$p < 0.05$ compared to the nonshocked group.

A: Bed nucleus of the stria terminalis



B: Amygdala



samples and those located caudally in the BST samples. The proCRF mRNA levels were found to be significantly different between groups in the amygdala ($F_{(2,35)}$ = 3.338, $p < 0.05$; Fig. 14B) but not in the BST ($F_{(2,33)} = 0.141$, $p = 0.869$; Fig. 14A). The Dunnett's T3 test revealed that the HR group had increased proCRF mRNA levels relative to nonshocked rats ($p < 0.05$) in the amygdala. The differences in proCRF mRNA levels between HR and LR did not reach significance ($p = 0.157$). There was no correlation between proCRF mRNA levels and freezing in the contextual fear test ($r^2 = 0.267$, $p = 0.105$) whereas there was a correlation between freezing to the tone ($r^2 = 0.376$, $p = 0.02$). Correlational analysis between proCRF mRNA levels and the latency in the elevated T-maze was not possible because of the non-parametric nature of the data from the elevated T-maze test.

3.6. Changes of Contextual Fear and Anxiety Levels After Footshocks

3.6.1. Contextual Fear to Shock Chamber

The two-way ANOVA on freezing in rats tested in the shock context revealed a main effect for "shock" ($F_{(1, 48)} = 1.444E3$, $p < 0.001$; Fig. 15) which showed that shocked rats had significantly higher level of contextual freezing than nonshocked rats ($p < 0.001$) but there was no difference in the level of freezing between different time points.

Fig. 15. Contextual fear to shock chamber at different time after the footshocks. Shocked rats showed significantly higher freezing time percent compared to nonshocked rats at 1 day post-shock (Early), 2 weeks post-shock (Middle) and 4 weeks post-shock (Late). The values in the histograms are mean ± SEM. ***p < 0.001 compared to the nonshocked group.

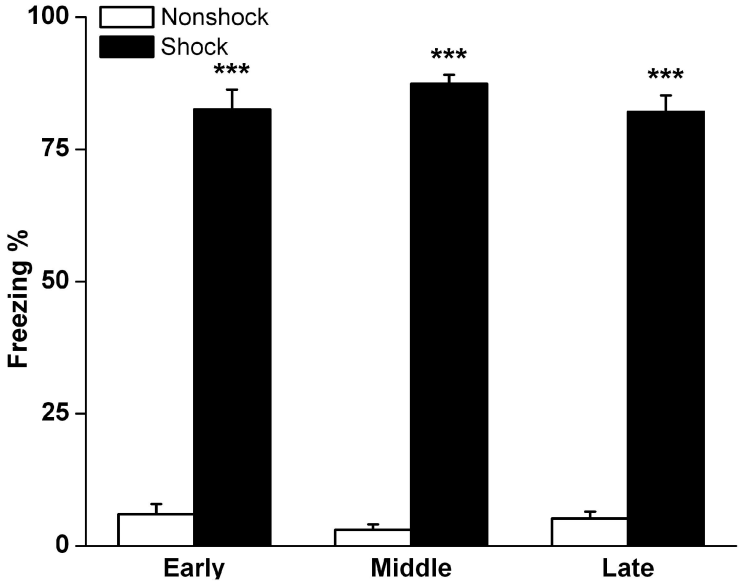
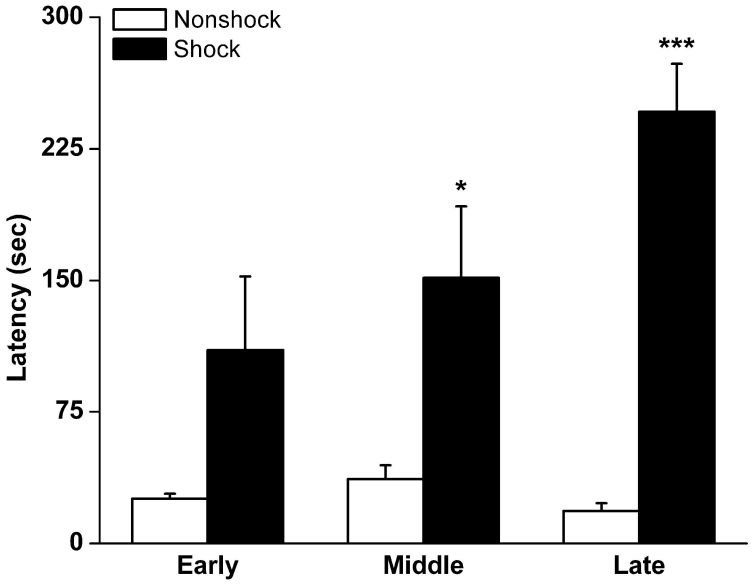

Nonshock
Shock
100
75
50
25
0
Freezing %
***
***
***
Early
Middle
Late

Fig. 16. Anxiety level in the social interaction test at different time after the footshocks. Shocked rats showed significantly higher anxiety level compared to nonshocked rats at 2 weeks post-shock (Middle) and 4 weeks post-shock (Late). The values in the histograms are mean ± SEM. *$p < 0.05$, ***$p < 0.001$ compared to the nonshocked group.


Nonshock
Shock
300
225
150
75
0
Latency (sec)
*
***
Early
Middle
Late

3.6.2. Anxiety Level in the Social Interaction Test

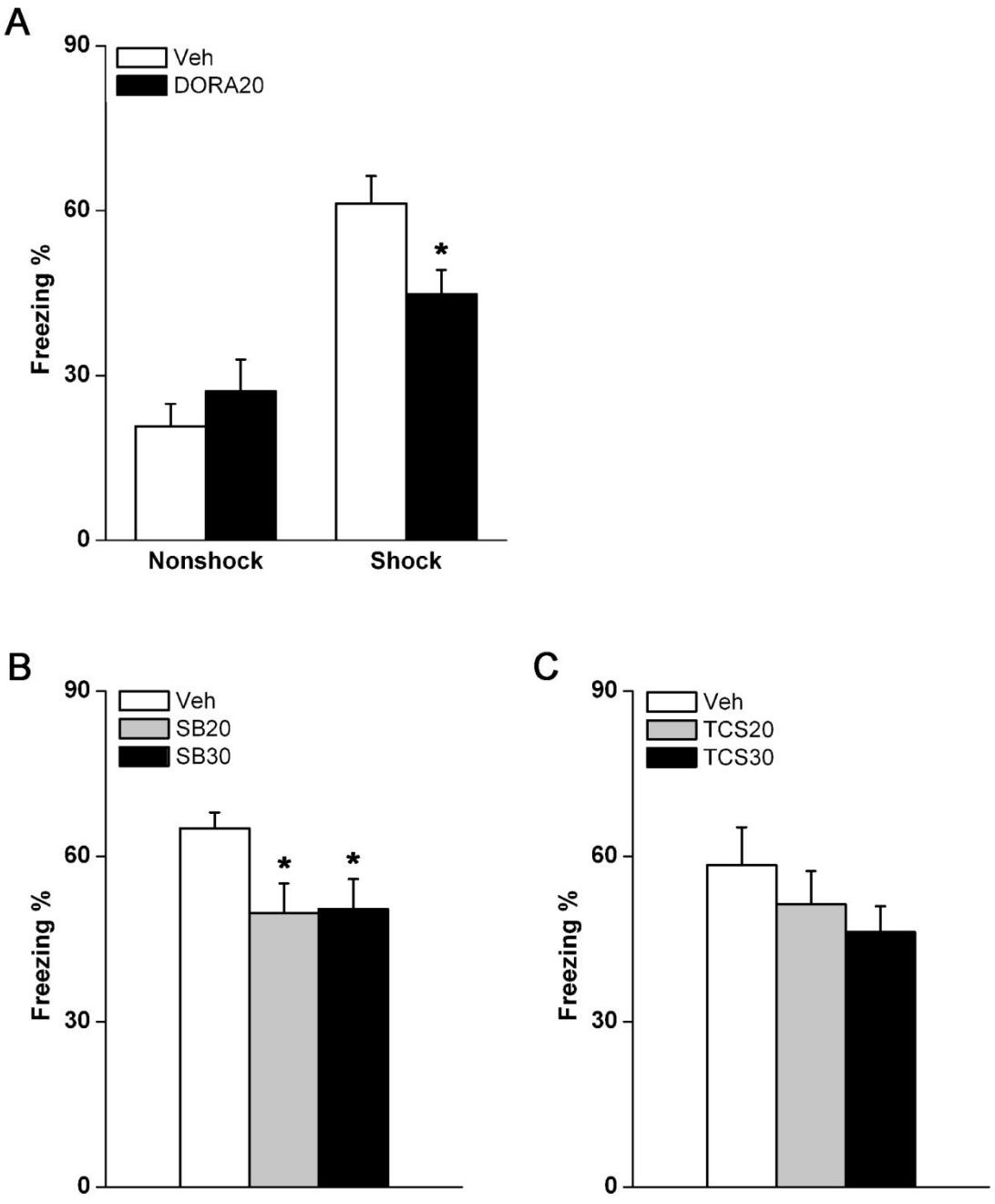
For the social interaction test, two-way ANOVA revealed a main effect for "shock" ($F_{(1, 48)} = 34.06$, $p < 0.001$; Fig. 16) and an interaction effect between "shock" and "time" ($F_{(2, 48)} = 3.19$, $p = 0.05$; Fig. 16) on latency going into the social zone. Similar to the freezing results, shocked rats had significantly longer latency going into the social zone than nonshocked rats ($p < 0.001$). The post hoc test showed that shocked rats took significantly longer latency to leave the safety of the dark box at 2 weeks post-shock ($p < 0.05$) and one month post-shock ($p < 0.001$) than nonshocked rats. These results suggested that it took some time for the anxiety to develop and shocked rats showed significantly higher levels of fear and anxiety at 2 weeks after the footshocks.

3.7. Effects of Orexin Receptor Antagonists on Contextual Fear Conditioning and Anxiety Induced by Footshocks

3.7.1. Effects of Orexin Antagonists on Contextual Fear to Shock Chamber

The two-way ANOVA revealed a main effect for "shock" ($F_{(1, 36)} = 32.24$, $p < 0.001$; Fig. 17A) and an interaction effect between "shock" and "dual orexin antagonist" ($F_{(1, 36)} = 4.96$, $p < 0.05$; Fig. 17A) on freezing in rats tested in the shock context at 14 days post-shock. Simple main effects analysis showed that shocked rats had significantly higher level of contextual freezing than nonshocked rats ($p < 0.001$) but there was no difference in the level of freezing between nonshocked rats given TCS1102 or vehicle. The post hoc Dunnett test showed that shocked rats that

Fig. 17. Effects of dual orexin receptor antagonist TCS1102 (DORA), orexin 1 receptor antagonist SB334867 (SB) and orexin 2 receptor antagonist TCSOX229 (TCS) on freezing to the shock chamber. (A) The dual orexin receptor antagonist TCS1102 at a dose of 20 mg/kg (DORA20, n = 10) decreased contextual freezing of shocked compared to vehicle (Veh) treated shocked rats (n = 8). (B) The orexin 1 receptor antagonist SB334867 at doses of 20 and 30 mg/kg (SB20 and SB30, n = 12 to 14) decreased freezing compared to vehicle treated rats (n = 15). (C) The same doses of the orexin 2 receptor antagonist TCSOX229 (TCS20 and TSC30, n = 12 to 14) had no significant effect on freezing compared to vehicle treated rats (n = 6). The values are expressed as mean ± SEM. *$p < 0.05$ compared to the shocked rats with vehicle injection.


A
90
60
30
0
Freezing %
Veh
DORA20
*
Nonshock
Shock
B
90
60
30
0
Freezing %
Veh
SB20
SB30
*
*
C
90
60
30
0
Freezing %
Veh
TCS20
TCS30

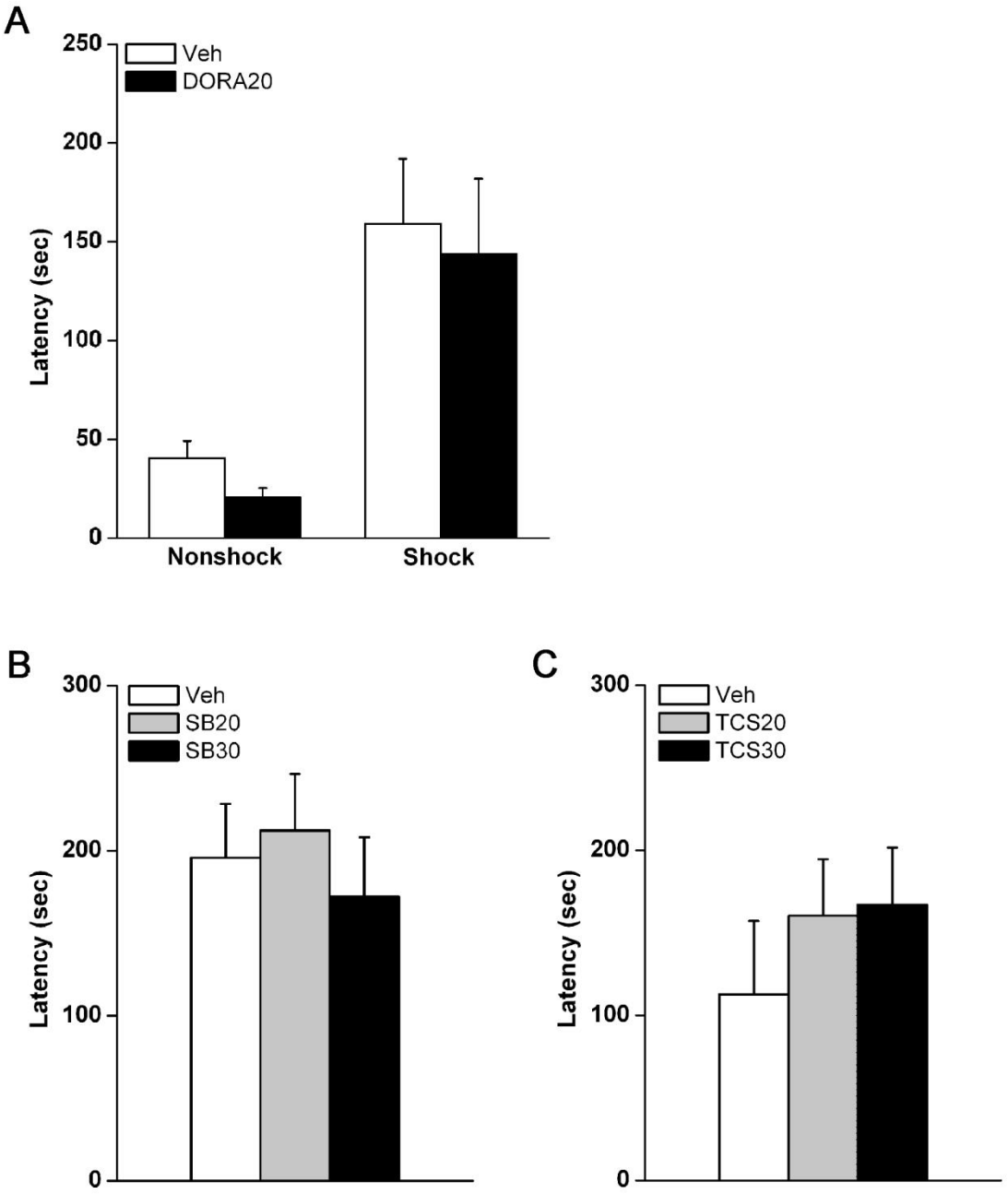
received the dual orexin antagonist TCS1102 at a dose of 20 mg/kg had a significantly lower level of contextual freezing compared to the shocked rats which were received the injection of vehicle ($p < 0.05$). The specific antagonists showed different effects on contextual freezing in shocked rats. The shocked rats treated with the OX1R antagonist SB334867 was found to be significantly different between the 3 dose groups ($F_{(2, 38)} = 3.71$, $p < 0.05$; Fig. 17B) and the post-hoc Dunnett test indicated that SB334867 decreased freezing when given at doses of 20 mg/kg ($p < 0.05$) and 30 mg/kg ($p < 0.05$) compared to vehicle treated shocked rats. In contrast, the same doses of the OX2R antagonist TCSOX229 (Fig. 17C) had no effect despite showing a trend towards decreasing freezing.

3.7.2. Effects of Orexin Antagonists on Anxiety Level in the Social Interaction

There was only a main effect for "shock" ($F_{(1, 44)} = 19.70$, $p < 0.001$; Fig. 18A) on latency in rats examined in the social interaction test at 14 days post-shock by the two-way ANOVA. It took significantly longer latency for the shocked rats to enter the social zone compared to nonshocked rats ($p < 0.001$) but shocked and nonshocked rats that received the dual orexin antagonist TCS1102 at a dose of 20 mg/kg showed similar level of latency to the rats which were received the injection of vehicle.

Consistent with these results, the specific antagonists showed no effect on anxiety examined in the social interaction test. The shocked rats treated by either the OX1R antagonist SB334867 (Fig. 18B) or the OX2R antagonist TCSOX229 (Fig. 18C) were not found to be significantly different between the 3 groups on latency.

Fig. 18. Effects of dual orexin receptor antagonist TCS1102 (DORA), orexin 1 receptor antagonist SB334867 (SB) and orexin 2 receptor antagonist TCSOX229 (TCS) on latency in the social interaction test. (A) Rats treated with the dual orexin receptor antagonist TCS1102 at a dose of 20 mg/kg (DORA20, n = 10) showed similar level of latency compared to rats treated with vehicle (Veh, n = 8). (B & C) The orexin 1 receptor antagonist SB334867 at doses of 20 and 30 mg/kg (SB20 and SB30, n = 12 to 14) and the same doses of the orexin 2 receptor antagonist TCSOX229 (TCS20 and TSC30, n = 12 to 14) had no significant effect on latency to enter the social zone compared to vehicle treated rats (n = 6 to 15). The values are expressed as mean ± SEM.


A
250
200
150
100
50
0
Latency (sec)
Veh
DORA20
Nonshock
Shock
B
300
200
100
0
Latency (sec)
Veh
SB20
SB30
C
300
200
100
0
Latency (sec)
Veh
TCS20
TCS30

Chapter 4

Discussion

4.1. Effects of Orexins Microinjections in the PVT on Anxiety Response

The evidence in support of a role for orexins in brain arousal has accumulated to the point where the modulation of arousal states is often seen as the most important function ascribed for these peptides (Sakurai 2007, Siegel 2004, Taheri, Zeitzer, and Mignot 2002). The present study extend these findings by showing that microinjections of orexin-A and orexin-B in the midline thalamus produced a form of emotional arousal in rats tested in an anxiety-provoking environment. This was shown by a decrease in open arm time and open arm entries in the elevated plus maze following microinjections in the PVT region. Microinjections of orexins in the PVT region were also found to increase ethological behaviors expressed during an approach-avoidance conflict and in anxiety-provoking situations (face grooming, SAP, and scanning time) (Albrechet-Souza et al. 2007, Cruz, Frei, and Graeff 1994, Garcia et al. 2005, Rodgers and Johnson 1995).

The dorsal and midline region of the posterior thalamus is composed of several small nuclei which could potentially be involved in the effects of orexins observed in the present study. The PVT, mediodorsal, intermediodorsal, centromedial, paracentral nuclei, as well as the habenula, are all within the general region where the orexin agonists or antagonists were injected in the midline thalamus. However, several facts suggest that the behavioral effects of orexins were largely mediated by

actions on the PVT. Compared to other thalamic nuclei, the PVT contains a high density of orexin fibers, which implies that this nucleus is one of the most important targets for these peptides (Kirouac, Parsons, and Li 2005). In addition, the PVT contains a moderate to high expression of mRNA for the OX1R and OX2R, respectively (Marcus et al. 2001). The centromedial nucleus, which is located approximately 1 mm ventral to the PVT, also contains a moderate signal for orexin receptor mRNA (Marcus et al. 2001). On the other hand, it is unlikely that a sufficient amount of the orexin peptide would have diffused to this nucleus to produce behavioral effects (Thorpe et al. 2003). The fact that injections of orexin-A in the lateral thalamus produced no effect in the elevated plus maze is consistent with this idea of limited diffusion and supports the conclusion that the orexin agents acted primarily at the PVT. The intermediodorsal nucleus, which is adjacent to the PVT, contains a very weak mRNA signal for orexin receptor, whereas the mediodorsal nucleus and the habenula do not express mRNA for the orexin receptors (Marcus et al. 2001). While it is not possible to completely exclude a contribution by other midline nuclei in the present experiment, the PVT appears to be the most likely candidate for mediating the behavioral effects of orexin receptor agonists observed here.

The strategy of microinjecting a peptide directly into the brain to examine its behavioral effects represents a means of understanding the function of an endogenously released peptide. This experimental approach has been used to provide evidence for orexins in the regulation of arousal and food intake (Espana, Plahn, and Berridge 2002, Jones et al. 2001, Nakamura et al. 2000, Rodgers et al.

2000, Thorpe and Kotz 2005, Thorpe et al. 2003). Here the study showed that microinjections of orexin-A and orexin-B in the PVT region at concentrations of 3.0 and 10.0 μg led to a decrease in the time spent in the open arm of the elevated plus maze, which is considered a behavioral response indicative of an anxiety state (Rodgers and Dalvi 1997, Walf and Frye 2007). While a previous study in our laboratory reported that microinjections of orexins in the PVT inhibited locomotion in some situations (Li et al. 2009), it is unlikely that a decrease in the exploration of the open arm in orexin-treated rats was due to an impairment of movement because the number of closed arm entries was not affected. Furthermore, administrations of orexins in the PVT increased the amount of grooming, SAP and scanning observed in the elevated plus maze. These ethological behaviors are expressed when an animal experiences anxiety in the presence of an approach-avoidance conflict (Albrechet-Souza et al. 2007, Garcia et al. 2005, Rodgers and Johnson 1995, Spruijt, van Hooff, and Gispen 1992). The fact that behaviors like grooming and SAP were increased in orexin-treated rats also indicates that these rats did not have motor deficits that could account for their lack of exploration in the open arm.

A plausible interpretation of the present experiment is that the level of anxiety expressed in a novel situation can be modulated by orexins released in the PVT. This is consistent with anatomical evidence showing that the PVT preferentially and heavily innervates key regions of the extended amygdala involved in regulating emotions and anxiety (Li and Kirouac 2008). As such, activation of orexin receptors in the PVT could enhance the excitability of neurons in the extended amygdala that

control anxiety levels. An alternative interpretation is that the anxiety-like response produced by injections of the orexins at the midline thalamus may have been due to an increase in generalized brain arousal. This does not appear to be the case because orexin administrations in the thalamus did not have any effect on locomotor activity, which is normally elevated in animals in heightened states of arousal (Pfaff et al. 2008). In fact, a study from our laboratory previously reported that microinjections of orexins in the PVT produced a weak inhibition of locomotion while increasing grooming and freezing (Li et al. 2010). A more likely explanation is that administrations of orexins in the PVT may have produced arousal effects that are specific to neurons that regulate emotional behaviors like anxiety. This interpretation is consistent with anatomical studies showing that the PVT sends an impressive projection to regions of the extended amygdala that densely stain for CRF and dynorphin (Li and Kirouac 2008), two peptides linked to the regulation of emotions and anxiety (Bals-Kubik et al. 1993, Bruchas et al. 2007, Bruchas, Xu, and Chavkin 2008, Knoll et al. 2007, Land et al. 2008, Lee and Davis 1997, Newton et al. 2002, Sahuque et al. 2006, Walker et al. 2009, Walker, Miles, and Davis 2009, Wittmann et al. 2009).

4.2. Effects of CRF and Kappa Opioid Receptor Blockade on Anxiety Produced by Orexin-A Microinjection in the PVT

In the present experiment, the microinjection of orexin-A in the PVT produced anxiety-like behaviors in the elevated plus maze and that the results are consistent

with the previous experiment. It was also showed that these behavioral effects appear to be mediated by the brain opioid and CRF systems by showing that intracerebroventricular (i.c.v.) injections of antagonists to kappa opioid or CRF receptors attenuated the anxiogenic effects induced by orexin-A. The results presented in this study indicate that endogenously released orexins may act on the PVT to regulate anxiety levels through mechanisms involving kappa opioid and CRF receptors.

The PVT is the only member of the midline and intralaminar thalamic nuclei to provide a direct and significant innervation to dynorphin and CRF rich regions of the extended amygdala (Li and Kirouac 2008). Dynorphin and CRF peptides, which are located in subpopulations of neurons in the extended amygdala, have been shown to play a key role in regulation of emotional behaviors (Bruchas, Xu, and Chavkin 2008, Davis 1998, Heinrichs and Koob 2004, Lang and Davis 2006). Results from the present experiment show that blocking CRF or kappa opioid receptors attenuated the behavioral effects of administrations of orexin-A in the midline thalamus. While these results suggest a possible link between the PVT and the CRF/dynorphin systems, more experiments will be required to show this unequivocally. Furthermore, a possibility cannot be excluded that the CRF and kappa antagonists could have decreased the brain arousal in a nonspecific manner, which in turn could have reduced the effectiveness of orexin-A to elicit anxiety. As shown in this study, this does not appear to be the case since the CRF and kappa antagonists given alone had no apparent effects on behaviors tested in the elevated plus maze. Clearly, more

experiments will be required to determine the role of these systems on anxiety and arousal.

4.3. Long-Lasting Changes of the Orexin System in Rats Exposed to Footshocks

Long-lasting changes of the orexin system were examined to determine the role of the orexin peptides and receptors on anxiety and arousal in a rat model of unpredictable footshocks which has been validated by previous studies of our laboratory (Chen et al. 2012, Chen et al. 2013, Rogala et al. 2012). In order to exclude the effects of behavioral tests on biochemical tests, there were no other behavioral tests after the footshocks in the present experiment. The results showed that the mRNA levels for ppOX and the OX1R as well as the protein expression level of the OX1R were elevated in the posterior hypothalamus of shocked rats at two weeks post-shock.

A number of studies have shown that the activity of the orexin neurons were elevated immediately after the exposure to distinct acute and chronic stress (Espana, Valentino, and Berridge 2003, Furlong et al. 2009, Ida et al. 2000, Nollet et al. 2011, Sakamoto, Yamada, and Ueta 2004, Zhu et al. 2002). For example, there was a significant increase in c-Fos expression of orexin neurons in rodents exposed to various type of stress, including novel environment, chronic mild stress, footshocks and conditioned fear context (Espana, Valentino, and Berridge 2003, Furlong et al. 2009, Nollet et al. 2011, Sakamoto, Yamada, and Ueta 2004, Zhu et al. 2002). In addition, it has been shown that the ppOX mRNA level was increased after 1 h of

immobilization stress (Ida et al. 2000). The present experiments extended these findings by examining the long-lasting changes (2 weeks) of the orexin system after the footshock exposure. The results are consistent with the hypothesis that the baseline activity of orexin neurons is enhanced following an episode of footshocks.

Previous study in our laboratory showed that the ppOX mRNA level was increased in the hypothalamus of shocked rats using in situ hybridization at 6 and 14 days after exposure of rats to footshocks (Chen et al. 2013). This study showed a significant increase of the ppOX mRNA level in the shocked rats using RT-PCR as well. However, the increase does not appear to be related to an enhanced activation of orexin neurons to the shock context or stress since the number of c-Fos expressing orexin neurons was not increased in shocked rats exposed to the shock chamber or a novel environment (Chen et al. 2013). In the present study, an upregulation of the OX1R as shown by mRNA and protein levels in the hypothalamus of shocked rats was found. In contrast, changes in orexin receptors in the PVT or the locus ceureolus/parabrachial area were not found. This suggests that the footshocks induced long-lasting changes in orexin activity and signaling possibly through receptor mechanisms involving the OX1R. Consequently, experimental evidence indicates that orexins act via the OX1R in the hypothalamus or possibly other unidentified brain regions to regulate the behaviors after the footshocks, such as anxiety and fear.

4.4. Long-Lasting Changes of the ppOX mRNA Level in a Modified Footshock Model of PTSD

Experiments were done to further examine the changes in the mRNA levels associated with the synthesis of orexins in a modified footshock model of PTSD (Chen et al. 2012). In HR, a subgroup of shocked rats with high levels of anxiety, significantly higher level of ppOX mRNA was found at 14 days post-shock compared to LR and nonshocked rats. The results are consistent with the previous experiments that footshock exposure leads to an elevated baseline production of orexins (Chen et al. 2013). In particular, the results suggest that the upregulation of the orexin system could be an important factor for anxiety state in the HR group.

Previous studies in our laboratory provided evidence that the acute fear response (one day after the footshocks) to a novel tone in a novel environment may predict the anxiety-like behaviors behaviors displayed later on (Chen et al. 2012, Chen et al. 2013). Shocked rats that showed an enhanced acute fear response (HR) displayed higher level of anxiety than shocked rats with low level of acute fear (LR) and nonshocked rats (Chen et al. 2012, Chen et al. 2013). This modified footshock model of PTSD highlights the importance of individual differences during the development of PTSD-like symptoms and the key role of hyperarousal in the peritraumatic period in the prediction of subsequent avoidance and anxiety.

In the present experiment, the baseline activity of orexin neurons was found to be elevated specifically in the HR, a group of shocked rats which exhibited high level of arousal at one day after the footshocks. The anxiety and fear levels of the rats were

not examined using behavioral tests to exclude the potential effect of these tests on the level of ppOX mRNA. According to the previous studies, the HR should show significantly higher level of anxiety than the LR and nonshocked rats at the same time point (14 days post-shock) (Chen et al. 2012, Chen et al. 2013). The results indicate that the high level of synthesis of orexins could be the main factor in elevating the arousal level of the HR. Therefore, it can be inferred that the upregulation of orexin system may enhance the arousal level in the HR which in turn lead to the development of subsequent anxiety and avoidance symptoms of PTSD.

4.5. Effects of Footshocks on Anxiety-Like Behaviors and mRNA Levels of Precursor Peptides for CRF and Opioids in the Forebrain

The purpose of this experiment was to evaluate whether neuropeptides in the extended amygdala and striatum implicated in the acute negative emotional reaction to a stress showed long-term changes that coincide with anxiety. The exposure of rats to unpredictable and moderately intense footshocks produced behavioral and biochemical changes that lasted for over two weeks in a subset of shocked rats. First, HR exhibited higher level of anxiety than LR and nonshocked rats. Second, enhanced level of CRF mRNA was detected in the amygdala of the HR while CRF, dynorphin and enkephalin mRNA levels were unchanged in the striatum and the rest of the extended amygdala.

The behavioral responses of rats exposed to a single episode of 1.5 mA footshocks were consistent with previous studies (Chen et al. 2012, Chen et al. 2013).

All shocked rats showed a similar amount of fear to the shock chamber but HR exhibited much higher level of anxiety than LR and nonshocked rats at two weeks post-shock. Indeed, HR took a longer time to exit the safety of the closed arm during the baseline trial of the elevated T-maze test showing that these rats had a higher level of fear to open spaces and height. The long-lasting anxiety exhibited by HR highlights the fact that there are individual differences in how rats react to moderately intense footshocks. As previously described, subsequent trials in the elevated T-maze produced avoidance of the open arm in both the LR and nonshocked rats (Chen et al. 2013, Graeff, Netto, and Zangrossi 1998, Zangrossi and Graeff 1997). Escape responses on the elevated T-maze were similar for all groups suggesting that the behavioral responses in HR do not represent helplessness (Chen et al. 2013, Graeff, Netto, and Zangrossi 1998, Zangrossi and Graeff 1997). Another purpose of the experiment was to evaluate potential neuropeptide contributors in the striatum and extended amygdala to the anxiety-like behavioral responses in rats exposed to a single episode of moderately intense footshocks.

Recent evidence points to the possibility that activation of dynorphin neurons in the NAc contributes to negative emotional states to acute stress (Bals-Kubik et al. 1993, Bruchas et al. 2007, Newton et al. 2002). Reports that the level of dynorphin mRNA is increased in the shell of the NAc of rats vulnerable to social defeat stress 24 hours after the last defeat episode (Berube et al. 2013) and that blockade of kappa opioid receptors 2 days after footshock exposure attenuated anxiety-like behaviors two weeks later points to the possibility that stress exposure could cause long term

anxiety-like behavior via enhanced dynorphin synthesis and release (Rogala et al. 2012). However, this experiment failed to find evidence that a single episode of footshock stress leads to a long-lasting increase in dynorphin activity in the striatum or the extended amygdala of shocked rats with high anxiety.

Stress-induced changes in enkephalin mRNA level have also been investigated as a possible mechanism involved in producing behavioral changes. For instance, the proenkephalin (proENK) mRNA level was found to be decreased in the basolateral amygdala at 24 hours after exposure to an unfamiliar rat in a social defeat model or predator odor (Berube et al. 2013, Hebb et al. 2004) and 7 days after exposure to a chronic unpredictable stress (Berube et al. 2014). In addition, enkephalin knockdown in the basolateral amygdala induced increased anxiety-like behaviors in social interaction and elevated plus maze test indicating that a possible attenuation in enkephalin activity may have supported anxiety following stress (Berube et al. 2014). However, the results of the experiment failed to find long-term changes in the level of proENK mRNA between nonshocked rats, LR and HR indicating that an attenuation of enkephalin activity was unlikely to be involved in the anxiety displayed by the HR.

Neurons that produce CRF are located in the amygdala and BST (Becker et al. 2007, Swanson et al. 1983). Extra hypothalamic neurons that produce CRF are activated by stress and have been found to be involved in some of the behavioral responses associated with stress (Aubry 2013, Heinrichs and Koob 2004). The mRNA level of CRF was found to be increased in the amygdala of HR two weeks

after the footshock exposure whereas the CRF mRNA level was unchanged in the BST region. These results indicate that footshocks could result in a long-lasting activation of CRF neurons in the amygdala in a way that potentially contributes to anxiety. This is in line with other studies that have reported that the CRF mRNA level was increased in CeA neurons of animals immediately after being exposed to stress (Hatalski, Guirguis, and Baram 1998, Hsu et al. 1998, Makino et al. 1999). Anatomical studies show that CRF neurons in the CeA project heavily to the BST (Alheid 2003, Alheid and Heimer 1988, Davis et al. 2010) and experimental evidence indicates that a projection from CRF neurons in the CeA to the BST plays a key role in anxiety (Davis et al. 2010, Walker and Davis 2008, Walker, Miles, and Davis 2009, Walker, Toufexis, and Davis 2003). For example, infusions of a CRF receptor antagonist into the BST decreased social defeat behavior (Jasnow, Davis, and Huhman 2004) and lesions of BST but not the CeA blocked CRF-enhanced startle (Lee and Davis 1997). As such, in a subgroup of shocked rats, shock exposure could result in anxiety because of an enhanced release of CRF in the BST produced by an increase in the activity of CRF neurons in the CeA. Another possible mechanism may involve CRF neurons in the basolateral amygdala. For example, the number of CRF-immunoreactive neurons in the basolateral amygdala were increased after neonatal separation stress (Becker et al. 2007) and pharmacological evidence showed that activation of CRF receptors in this region of the amygdala increased anxiety-like behaviors (Rainnie et al. 2004).

Prolonged changes in the levels of neuropeptides associated with negative emotional states represent a plausible mechanism for mediating the long-lasting anxiety that results from a single episode of footshocks (Chen et al. 2013, Landgraf 2001). A contribution of CRF neurons in the amygdala to this process would be consistent with research showing that this CRF system is activated by stress and contributes to anxiety-like behaviors in a variety of experimental conditions. While not investigated in the present experiment but as indicated by other investigations (Bruchas et al. 2009, Hendriksen et al. 2012, Sztainberg et al. 2010), it is possible that changes in expression of the CRF1 receptor in the amygdala or BST may also contribute to anxiety-like behaviors following footshocks.

4.6. Changes of Contextual Fear and Anxiety Levels After Footshocks

To choose the appropriate time point for pharmacological experiments, a pilot experiment was done to examine the development of contextual fear and social avoidance over several weeks in a rat footshock model of PTSD. The shocked rats exhibited high level of fear to the chamber where they received the footshocks at 1 day, 2 weeks and 4 weeks post-shock without any significant rise or decline. In the social interaction test, the shocked rats showed significant higher anxiety compared to nonshocked rats at 2 weeks and 4 weeks post-shock whereas there was no significant difference in anxiety in shocked and nonshocked rats at 1 day post-shock.

Similar with previous studies, the footshocks induced long-lasting changes of fear-like and anxiety-like behaviors in rats (Chen et al. 2012, Chen et al. 2013, Rogala

et al. 2012). The present study also showed the different time courses of contextual fear and avoidance to another unfamiliar rat after exposure to relatively intense footshocks. The conditioned fear of the shocked rats to the shock context appeared immediately after the footshocks and remained at a high level until at least 4 weeks post-shock. Meanwhile, the avoidance to an unfamiliar rat (a measure of social anxiety) was not seen the day after the footshocks but observed at 2 weeks post-shock. These results indicate that avoidance appear later after the fear experience of footshocks. As mentioned in the introduction section, the various symptoms of PTSD in humans exposed to trauma are found to have different development courses (North 2001, Schell, Marshall, and Jaycox 2004). For example, re-experiencing appears immediately after the exposure of trauma (North 2001, Schell, Marshall, and Jaycox 2004). In contrast, avoidance usually needs an incubation period and appears later when PTSD is often diagnosed (North 2001, Schell, Marshall, and Jaycox 2004). The results of the present animal experiment are consistent with the clinical observations of human individuals with PTSD.

4.7. Effects of Orexin Receptor Antagonists on Contextual Fear Conditioning and Anxiety Induced by Footshocks

The purpose of this experiment was to determine the role of orexin receptors in contextual fear conditioning and anxiety. A systemic injection of the OX1R antagonist SB334867 or the dual orexin receptor antagonist TCS1102 reduced the fear to the shock context. In contrast, all the drugs tested, including the dual and single

orexin receptor antagonists, had no significant effect on alleviating the avoidance to a novel rat. These results are consistent with the hypothesis that the orexin system plays a role in mediating the freezing associated with contextual fear and that the OX1R could be the main receptor involved in orexins' contribution to fear.

The dual orexin receptor antagonist TCS1102 reduced contextual fear which is in line with other studies reporting that dual orexin antagonists decreased freezing and the cardiovascular responses associated with contextual fear (Chen et al. 2013, Furlong et al. 2009). It was also observed that the OX1R antagonist SB334867 decreased fear to the shock context, a result that is consistent with the previous biochemical experiments showing an increase in the expression of the OX1R in the hypothalamus of shocked rats. Meanwhile, the same doses of the OX2R antagonist TCSOX229 had no effect on the contextual fear. The specific antagonists used in this experiment have similar binding kinetics and affinity to their respective receptor in that SB334867 has a 50-fold selectivity for OX1R compared to OX2R whereas TCSOX229 has a 250-fold selectivity for OX2R over OX1R (Duxon et al. 2001, Hirose et al. 2003, Mould et al. 2014). Consequently, the doses of TCSOX229 used in the present experiment should have produced comparable or better antagonism of the OX2R than SB334867 would have on the OX1R. Evidence from another study indicates that the involvement of a specific orexin receptor type may be dependent on the fear conditioning model used. The administration of the OX1R antagonist SB334867 in the lateral ventricles was reported to have no effect on the freezing to auditory cues that had been previously paired with footshocks (Sears et al. 2013).

Reports that contextual and cued fear were impaired in OX1R knockout mice whereas only contextual fear was impaired in OX2R knockout mice, indicate a potential difference between the effects of single injections of an orexin antagonist and the long-term changes that may be occurring in orexin receptor knockout models (Soya et al. 2013).

The effects of the dual orexin receptor antagonist or selective orexin receptor antagonists on avoidance to a novel rat were also investigated. Recent evidence points to the possibility that orexins participate in the regulation of anxiety-like behaviors (Chen et al. 2013, Dong, Li, and Kirouac 2015, Khalil and Fendt 2017, Viviani et al. 2015). For example, orexin-deficient mice showed elevated anxiety in several behavioral paradigms, including open field, light-dark box and predator avoidance (Khalil and Fendt 2017). The dual orexin receptor antagonist TCS1102 was found to significantly reduce anxiety induced by footshocks in the open field and avoidance to open spaces and height of the HR in the elevated T-maze (Chen et al. 2013). However, not only the selective orexin receptor antagonists SB334867 and TCSOX229 but also the dual orexin receptor antagonist TCS1102 failed to alleviate the avoidance to a novel rat induced in the shocked rats. The inconsistence may be due to the distinct behavioral models used. A recent report that almorexant, a dual orexin receptor antagonist, attenuated avoidance in the elevated plus maze whereas had no effect on avoidance in the dark-light box, seems to support the view (Viviani et al. 2015).

In summary, pharmacological evidence from this experiment and other studies (Chen et al. 2013, Furlong et al. 2009) indicates that the systemic injection of a dual orexin antagonist decreased fear. The present experiment suggests that this effect is primarily mediated by an upregulation of orexin neuron activity through an OX1R mediated mechanism.

4.8. General Conclusions

It is well documented that orexins can produce a stress-like response by enhancing the activity of the HPA axis and the secretion of stress hormones in the circulation (Chang et al. 2007, Ida et al. 2000, Jaszberenyi et al. 2000, Kuru et al. 2000, Sakamoto, Yamada, and Ueta 2004, Samson et al. 2007, Samson et al. 2002). Orexin neurons are activated by novelty-stress, pain, and contextual cues associated with shock (Espana, Valentino, and Berridge 2003, Furlong et al. 2009, Watanabe et al. 2005, Winsky-Sommerer et al. 2004, Zhu et al. 2002), but not other stressors like exposure to cold and restraint (Furlong et al. 2009, Kiss 2007). Consequently, a relationship between the orexin peptides, arousal and stress is likely to exist, but the nature of this relationship remains poorly understood.

In the present thesis, the role of orexins and the orexin receptors in anxiety-like and fear-like behaviors as well as some of their potential underlying mechanisms were studied. The experiments showed that microinjections of orexins in the PVT region induced anxiety-like behaviors in the elevated plus maze and this anxiety could be blocked by i.c.v. administrations of CRF or kappa opioid receptor antagonists. It

was also found that the expression of ppOX and OX1R in the hypothalamus as well as the production of CRF in the amygdala of rats was upregulated 2 weeks after the exposure of a single episode of footshocks. In addition, pharmacological experiments showed that systemic injections of the dual orexin receptor antagonist TCS1102 or the OX1R antagonist SB334867 significantly reduced the fear of shocked rats to the shock context.

The PVT receives dense innervations from the orexin neurons and sends abundant projections to some brain regions mediating negative emotion states, including the NAc, BST and CeA (Li and Kirouac 2008, Nambu et al. 1999, Parsons, Li, and Kirouac 2006, 2007). This suggests that the PVT may be a pathway through which the orexins regulate the expression of emotions. The present thesis provides some evidence for the role of the orexin-PVT pathway in the anxiety-like behaviors of rats. The anxiogenic effect of orexin-A was blocked by antagonists of CRF or kappa opioid receptors. One possibility is that orexins activate neurons in the PVT that project to CRF and dynorphin rich regions of the NAc and extended amygdala. Consequently, the release of CRF and dynorphin, two peptides associated with anxiety, would exert their behavioral effects by activating CRF and kappa opioid receptors, respectively. This inference is supported by anatomical evidence that CRF and dynorphin are coexpressed in many neurons in the lateral CeA and that fibers from the PVT overlap with CRF neurons in the lateral CeA and dorsolateral BST (Li and Kirouac 2008, Marchant, Densmore, and Osborne 2007). Thus, results from experiments described in this thesis as well as studies from other groups indicate

that orexins could be one of the key modulators involved in producing symptoms of anxiety disorders (Furlong et al. 2009, Heydendael et al. 2011, Steiner, Lecourt, and Jenck 2012, Suzuki et al. 2005, Viviani et al. 2015).

A footshock model of PTSD was used to examine if the orexin neurons were activated for a prolonged period after exposure to a stressful stimulus like footshocks. It is well known that hyperarousal is a key feature of PTSD and this symptom is a good predictor for the subsequent development of other symptoms, especially the avoidance symptom (Marshall et al. 2006, Schell, Marshall, and Jaycox 2004, Solomon, Horesh, and Ein-Dor 2009, Thompson et al. 2004, Weems et al. 2003). The orexin system, an important arousal-promoting system, was found to be upregulated for over two weeks after footshock exposure. These findings along with other studies presented provide support for the view that enhanced activity of the orexin system contributes to the hyperarousal and anxiety seen after footshock exposure. Interestingly, the study showed that an upregulation of the orexin system specifically in a subgroup of shocked rats that showed high acute fear response to a novel stimulus. These HR rats showed anxiety-like and fear-like behaviors which resemble the vulnerable human individuals that go on to develop PTSD following exposure to a traumatic event. In addition, the production of the CRF in the amygdala was found to be upregulated in the HR. As indicated above, one possibility is that CRF neurons in the amygdala get activated because of the enhanced action of orexins on the PVT and the subsequent activation of CRF neurons through projections from the PVT to the amygdala (Kirouac 2015, Li and Kirouac 2008). It

has been shown that CRF neurons in the lateral CeA send fibers to the BST and that the terminals of these fibers act on CRF receptors on glutamate terminals to promote glutamate release in the BST (Davis et al. 2010, Walker and Davis 2008, Walker, Miles, and Davis 2009). The glutamate release is believed to activate the neurons in the BST which in turn mediate the specific autonomic and behavioral responses of anxiety (Davis et al. 2010, Walker and Davis 1997, 2008, Walker, Miles, and Davis 2009, Walker, Toufexis, and Davis 2003).

The current treatment for PTSD mainly includes psychotherapy and pharmacotherapy (Davidson et al. 2004, Pratchett et al. 2011, Warner et al. 2013). The most commonly used psychotherapy is cognitive behavioral therapy which is a category of interventions focused on changing irrational cognition and behaviors (Davidson et al. 2004, Pratchett et al. 2011, Warner et al. 2013). The interventions including exposure therapy, cognitive restructuring, psychoeducation, and relaxation techniques which can be used alone or in combination (Bisson and Andrew 2007, Davidson et al. 2004, Pratchett et al. 2011). In addition, other therapies including hypnosis, acupuncture, and yoga have also been reported to be potentially effective for PTSD treatment (Keane, Marshall, and Taft 2006, Warner et al. 2013).

The first-line medication choices for PTSD patients are selective serotonin reuptake inhibitors (SSRIs) (Keane, Marshall, and Taft 2006, Pratchett et al. 2011, Warner et al. 2013, Yehuda 2002). Sertraline and paroxetine, which are both SSRIs, are the only two medications approved for the treatment of PTSD by the Food and Drug Administration (FDA) in the U.S. (Keane, Marshall, and Taft 2006, Pratchett et

al. 2011, Warner et al. 2013). The specific mechanism by which the serotonin system modulates PTSD is not yet fully understood, but serotonin has been found to be involved in several symptoms of PTSD including arousal, depression and anxiety (Norman et al. 2012). SSRIs selectively block the reuptake of serotonin in the brain leading to elevated neurotransmission of serotonin system and indirect downregulation of beta-adrenoceptors (Heym and Koe 1988). Besides SSRIs, other antidepressants including tricyclic antidepressants and monoamine oxidase inhibitors may also be effective in the treatment of PTSD and can be used as second-line medication choices (Davidson et al. 2004, Keane, Marshall, and Taft 2006, Warner et al. 2013). In addition, because of the high rate of comorbid disorders in PTSD patients, medications like hypnotics, mood stabilizers, or anticonvulsants are also used in the treatment of PTSD and show efficacy for some target symptoms of PTSD (Davidson et al. 2004, Keane, Marshall, and Taft 2006, Pratchett et al. 2011, Warner et al. 2013).

As described above, a range of treatments have been developed for PTSD (Davidson et al. 2004, Keane, Marshall, and Taft 2006, Pratchett et al. 2011, Warner et al. 2013, Yehuda 2002). However, none of them have satisfactory treatment effects. The symptom improvement is not adequate for many individuals and a large proportion of patients do not benefit from the treatments (Davidson et al. 2004, Pratchett et al. 2011). In particular, the side effects and potential toxicity of some medications are high which could do more harm to the patient's health (Keane, Marshall, and Taft 2006). Consequently, it is vital to study the underlying

neurobiological basis for PTSD which could lead to the development of new and more effective treatments.

In this thesis, a role for orexins and the OX1R in contextual fear was found which suggests a potential application of orexin receptor antagonists in the treatment of disorders with high level of fear. In fact, there is emerging evidence indicating that the orexin system is involved in a number of pathologies associated with impaired fear processing, such as phobias, panic attacks and PTSD (Chen et al. 2013, Flores et al. 2015, Flores et al. 2014, Johnson et al. 2015, Johnson, Molosh, et al. 2012, Johnson, Samuels, et al. 2012, Johnson et al. 2010, Sears et al. 2013). Several antagonists for both of the orexin receptors, including TCS1102, almorexant and DORA-12, have been shown to have an effect on fear-like and anxiety-like behaviors in distinct animal models (Chen et al. 2013, Flores et al. 2015, Johnson et al. 2015, Viviani et al. 2015). It is well known that a dual orexin antagonist called suvorexant has been approved by the FDA of U.S. and Japan for the treatment of insomnia in 2014 (Sutton 2015, Yeoh et al. 2014). Thus, orexin antagonists could be good candidates for treatment of different psychiatric disorders associated with high level of fear and anxiety. The OX1R appears to be a good target for reducing fear-related behaviors and some anxiety disorders. However, more research will be required to determine how to apply orexin antagonists to treat psychiatric disorders like phobias, anxiety and PTSD.

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