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Invited review

The role of the orexin system in stress response

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HIGHLIGHTS

- Orexin neurons project throughout the entire brain and are involved in diverse behavioral and physiological functions.
- While the orexin system is activated in response to acute stress, the effects of chronic stress on the orexin system are variable.
- Manipulating orexin activity *in vivo* leads to alterations in behavioral and physiological functions relevant for the stress response.
- Orexins have also been implicated in mental health disorders which may be important for their therapeutic potential.

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ABSTRACT

Orexins are neuropeptides that are exclusively produced by hypothalamic neurons, which project throughout the entire brain. Orexin, also known as hypocretins, were initially identified to play a fundamental role in food intake, arousal and the regulation of sleep and wakefulness. Recent studies identified orexins to be critical for diverse physiological processes including motivation, reward, attention, emotional regulation, stress and anxiety. Here, I review recent findings that indicate orexin has an important role in acute and chronic stress. I also summarize the recent optogenetic and chemogenetic studies that have advanced our understanding of the orexin system. I will conclude by discussing clinical studies that implicate orexins in mental health disorders.

This article is part of the Special Issue entitled 'Hypothalamic Control of Homeostasis'.

1. Introduction

The neuropeptides orexin-A and orexin-B are produced in a small group of neurons located exclusively in the hypothalamus. Specifically, orexin-expressing neurons are found in the lateral hypothalamus (LH), the perifornical area (PFA) and the dorsomedial hypothalamus (DMH). Orexin-A and -B (also known as hypocretin-1 and -2) are produced from a common precursor peptide, prepro-orexin (prepro-hypocretin), which is encoded by the *hypocretin* (*Hcrt*) gene. Orexins were identified by two separate research groups to be endogenous ligands for two closely related orphan G protein coupled receptors (de Lecea et al., 1998; Sakurai et al., 1998). The receptors for orexin are differentially expressed throughout the brain (Marcus et al., 2001; Trivedi et al., 1998). Orexin-A binds to both orexin receptor 1 (OX1R, Hcrtr1) and orexin receptor 2 (OX2R, Hcrtr2) with equal affinity, whereas orexin B binds preferentially to OX2R.

Early studies examining the functions of orexins demonstrated an important role in nutritional homeostasis and appetite regulation (Sakurai et al., 1998). The infusion of orexins into the brain stimulates food intake (Dube et al., 1999; Sakurai et al., 1998; Sweet et al., 1999). Moreover, fasting induces upregulation in prepro-orexin mRNA levels (Cai et al., 1999; Sakurai et al., 1998). Shortly after the discovery of orexins, an additional role in sleep was demonstrated. The administration of orexin A into the lateral ventricles of rats induces wakefulness (Hagan et al., 1999). Conversely, the targeted disruption of the orexin gene in mice produces a narcoleptic phenotype (Chemelli et al., 1999). Canine narcolepsy has been associated with a mutation in the *hypocretin receptor 2* (*Hcrtr2)* gene (Lin et al., 1999). The detection of orexin deficiency in the cerebrospinal fluid (CSF) of patients with narcolepsy and identification of a link between early onset narcolepsy and a dominant mutation in *Hcrt* in humans reinforced the critical role of orexins in sleep regulation (Nishino et al., 2000). *In vivo* electrophysiological recordings showed that orexin neurons are silent during sleep and discharge during active waking. Their activity levels are moderate during grooming and eating but maximal during an exploration task that requires increased vigilance (Mileykovskiy et al., 2005). In parallel to their role in feeding, sleep/wakefulness and arousal, orexins were found to modulate motivation, reward, energy homeostasis and stress (for reviews see (Giardino and de Lecea, 2014; Harris and Aston-Jones, 2006; Johnson et al., 2012a; Sakurai, 2014, 2003).

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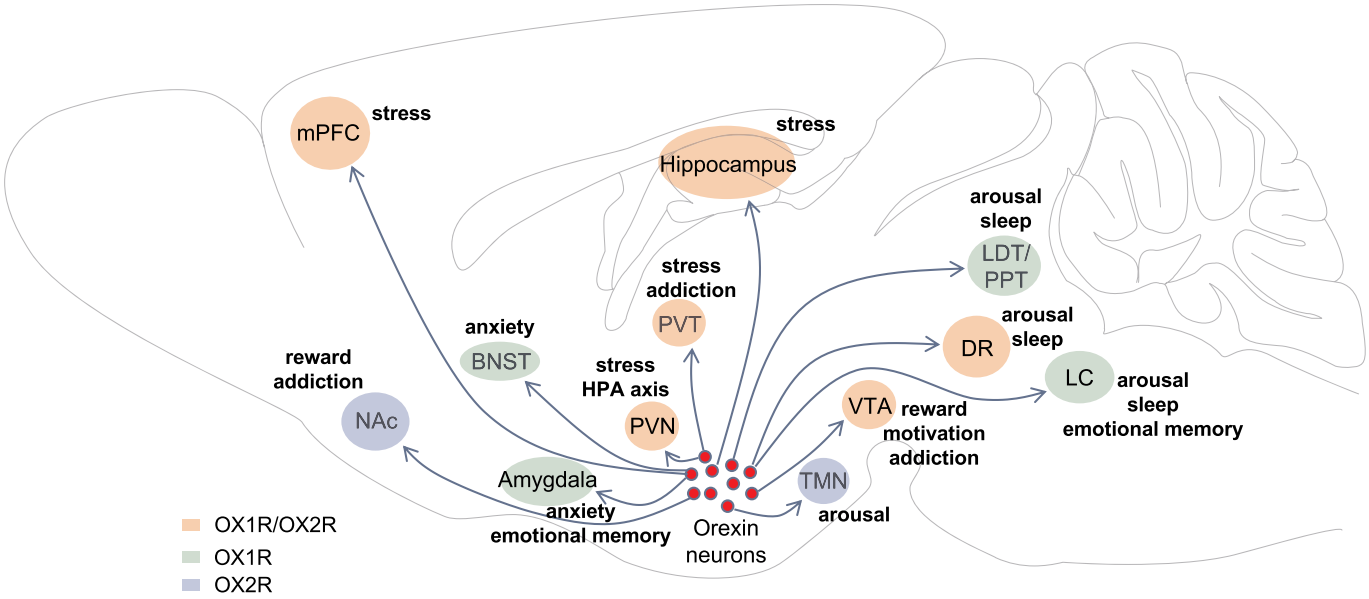

Fig. 1. Efferent projections of orexin/hypocretin neurons, the distribution of orexin receptors and the associated behavioral functions in postsynaptic target regions. Orexin neurons project widely throughout the brain onto target regions which include the ventral tegmental area (VTA), locus coeruleus (LC), dorsal raphe (DR), the laterodorsal tegmental nucleus and the pedunculopontine tegmental nucleus (LDT/PPT), tuberomammillary nucleus (TMN), hypothalamic paraventricular nucleus (PVN), paraventricular nucleus of the thalamus (PVT), bed nucleus of the stria terminalis (BNST), amygdala, nucleus accumbens (NAc), hippocampus and the medial prefrontal cortex (mPFC). The postsynaptic target sites of orexin neurons express either orexin receptor 1 (OX1R), orexin receptor 2 (OX2R) or a combination of these receptors (OX1R/OX2R). Each of these target regions is involved in the regulation of diverse behavioral and physiological functions.

In this review, I will give a general introduction of orexin projection sites and the involvement of orexin output regions in different behavioral functions. I will then review the effects of acute and chronic stress on the orexin system. This will be followed by the most recent studies reporting *in vivo* manipulations of orexin neuronal activity and the observed behavioral effects. Lastly, I will discuss the implications of the orexin system in mental health disorders.

2. Projections of orexin neurons

Although the number of orexin-producing neurons is relatively small (about 70,000 in the human brain and 3000 in the rat brain), they project to a large number of brain regions throughout the central nervous system including the brainstem, prefrontal cortex, ventral tegmental area, thalamus, locus coeruleus and the limbic system (Peyron et al., 1998). A variety of functions have been ascribed to the orexin system. This is likely due in part to connectivity with numerous different regions with diverse functions (Fig. 1). The densest connections from orexin neurons are the projections onto noradrenergic neurons of the locus coeruleus (LC) and histaminergic neurons of the tuberomammillary nucleus (TMN). These pathways are important for sleep and wakefulness (for reviews see (Berridge and Waterhouse, 2003; Lin, 2000; Thakkar, 2011). Additionally, orexin projections to cholinergic neurons in the basal forebrain have been implicated in attentional processing (Boschen et al., 2009), acquisition of an olfactory discrimination task (Piantadosi et al., 2015) and arousal (Arrigoni et al., 2010; Fadel and Frederick-Duus, 2008). Orexin also projects to the noradrenergic neurons of the pedunculopontine tegmental nucleus, and these connections are likely important for arousal (Mena-Segovia and Bolam, 2017). Although a direct association between orexin's effect on stress response and these projections remains to be elucidated, the physiological functions that they regulate are essential for adaptive stress responses.

Orexin neurons project abundantly to the ventral tegmental area (VTA) and nucleus accumbens (NAc) (Peyron et al., 1998). These regions are highly associated with reward and motivation-related behaviors (Wise, 1996). Orexin neurons are activated by cues indicating food and drug rewards (Boutrel et al., 2005; Harris et al., 2005). The injection of orexin A into the brain induces drug-seeking behaviors in extinguished rodents (Mahler et al., 2012) demonstrating that orexin is also involved in the reinstatement of reward-seeking behavior. The role of orexins in reward partly stems from their effects on VTA dopaminergic neurons. Orexins activate VTA dopaminergic neurons directly (Korotkova et al., 2003). Moreover, the orexin-induced potentiation of *N*-methyl-D-aspartate receptor (NMDAR)-mediated neurotransmission onto VTA dopaminergic neurons is critical for drug-induced synaptic plasticity (Baimel and Borgland, 2012; Borgland et al., 2009, 2006). The administration of OX1R antagonist SB334867 into the VTA blocks the potentiation of excitatory neurotransmission and the decrease in inhibitory neurotransmission induced by morphine treatment in rats (Baimel and Borgland, 2015). These studies suggest that orexin signaling is essential for drug-induced modulation of the excitatory and inhibitory balance onto VTA dopamine neurons. Intra-VTA injections of orexin also lead to an increase in the extracellular dopamine concentrations in NAc (España et al., 2011). On the other hand, several reports have also identified that orexin-induced signaling is important for stress-induced drug-seeking behavior (Boutrel et al., 2005; Schmeichel et al., 2017; Tung et al., 2016; Wang et al., 2009) suggesting that stress may itself play an important role in the orexin-induced reinstatement of reward-seeking behavior. Thus, an extensive amount of literature points to orexin acting as a positive modulator of the VTA dopaminergic reward system despite its role in promoting stress-related behaviors. This raises the notion that the orexin system is not associated with a particular valence but that its role is characterized by the functions of the target region.

Orexin neurons also send many projections to the dorsal raphe nucleus (DRN) (Lee et al., 2005; Peyron et al., 1998). Serotonin (5-HT)-producing neurons within the DRN are the principle targets of the most widely used antidepressants. These neurons are highly involved in the regulation of emotional memory. Orexin-immunoreactive fibers make direct synaptic contacts with the dendrites of the 5-HT neurons in the DRN (Wang et al., 2005). Electrophysiological recordings on DRN slices and *in vivo* showed that orexin has an excitatory effect on 5-HT neurons through signaling via both OX1R and OX2Rs (Brown et al., 2002, 2001;

Liu et al., 2002; Soffin et al., 2004; Takahashi et al., 2005) involving the opening of a sodium-dependent non-selective cation channel (Liu et al., 2002). The direct infusion of orexin peptides into the DRN leads to a local increase in 5-HT release (Tao et al., 2006). 5-HT neurons fire tonically during the awake state; their activity is reduced during slow wave sleep and completely ceases during REM sleep (Cespuglio et al., 1981; Daszuta et al., 1979; McGinty and Harper, 1976; Trulson and Jacobs, 1979). Interestingly, the targeted restoration of orexin receptors in DRN and the chemogenetic activation of DR 5-HT neurons prevented cataplexy-like episodes and narcolepsy in OXR KO mice (Hasegawa et al., 2014). The importance of both orexins and DR 5-HT neurons in the modulation of stress response and emotional behavior and the dense projections between these two systems suggest a causal link of orexin-DR circuitry for stress regulation. Indeed, chronic social isolation stress reduces the number of orexin-induced action potentials in DRN 5-HT neurons (Sargin et al., 2016). Further studies are required to determine the mechanisms of how stress affects orexin responses in DRN.

Orexin immunoreactive terminals are also found in brain regions important for the expression of fear and anxiety-related behaviors, including the central nucleus of the amygdala (CeA) and the bed nucleus of the stria terminalis (BNST). Orexin exerts excitatory effects on CeA neurons via OX2R (Bisetti et al., 2006) and on a subset of BNST neurons via OX1R (Lungwitz et al., 2012; Trivedi et al., 1998). The effects of orexin within these circuits are important for anxiety-related behaviors and will be reviewed below.

Local hypothalamic circuits, especially those between orexin and corticotropin-releasing factor (CRF) neurons, play an important role in the stress response. CRF is synthesized in the paraventricular nucleus (PVN) of the hypothalamus and activates the hypothalamic-pituitary-adrenal (HPA) axis. This leads to adrenocorticotropic hormone (ACTH) and corticosterone release during stress (Owens and Nemeroff, 1991). CRF directly depolarizes and increases the firing rate of orexin neurons via activation of the CRF receptor 1 in these neurons (Winsky-Sommerer et al., 2004). Orexin neurons project back to PVN cells that express both OX1R and OX2R (Cluderay et al., 2002; Hervieu et al., 2001; Trivedi et al., 1998), and PVN neurons are depolarized by orexin (Samson et al., 2002). Of note, PVN consists of a heterogeneous group of cells categorized into magnocellular neurons (oxytocin- and vasopressin-secreting cells) and parvocellular neurons (CRF, thyrotropin-releasing hormone, glutamate, GABA or somatostatin secreting cells) (Swanson and Sawchenko, 1983). Orexin interacts with both groups of neurons by altering their excitability, suggesting a functional connection between orexin and different populations of PVN neurons (Follwell and Ferguson, 2002; Samson et al., 2002; Shirasaka et al., 2001).

Given that orexin neurons are situated within the region of the brain critical for adaptive stress responses and that they project to other regions involved in stress, it is not surprising that orexins may play an important role in the regulation of the stress response. Evidence points to a critical role of hypothalamic orexin neuronal activity in the expression of stress-related behaviors and stress-induced anxiety-like responses.

3. Stress and the orexin system

3.1. Acute stress

The acute stress response is the body's natural reaction to threats and unpredictable occurrences in the environment. It increases arousal and prepares the body to cope in a survival situation. The hypothalamus, as part of the HPA system, is an integral component of the physiological stress response. The association between the hypothalamic orexin neurons and acute stress has been extensively studied. Below I will review the studies that investigated the orexin system in relation to different acute stress procedures.

Initial studies used direct administration of orexin-A and orexin-B into the brain to investigate the effects of these peptides on the stress response. The intracerebroventricular (icv) administration of orexin in rats increased stress-induced grooming and chewing behaviors (Espana et al., 2002; Ida et al., 1999) which were inhibited by the pre-administration of an antagonist for CRF (Ida et al., 2000). Orexin administration caused an increase in plasma adrenocorticotropic hormone (ACTH) and corticosterone levels (Kuru et al., 2000; Samson et al., 2002), and increased c-fos mRNA in PVN (Kuru et al., 2000; Sakamoto et al., 2004) indicating that orexin may potentiate stress responses. There are close anatomical interactions between PVN CRF axonal terminals and orexin perikaryal and dendrites in the lateral hypothalamus (Winsky-Sommerer et al., 2004). A large proportion of orexin neurons express CRF-R1/2 receptors and are depolarized in response to bath application of CRF. The acute stress-induced c-fos expression in orexin neurons is blunted in CRF-R1 KO mice (Winsky-Sommerer et al., 2004). Footshock stress and seizure-induced elevation in orexin mRNA can be counterbalanced by the blockade of CRF-R1 in the lateral hypothalamus in rats (Mokhtarpour et al., 2016). Therefore, the circuitry between the CRF and orexin neurons may play a significant role in the regulation of the hypothalamic response to acute stress.

Orexin mRNA expression is modulated by glucocorticoid levels and stress. Removal of circulating glucocorticoids as a result of adrenalectomy decreases orexin mRNA levels, which are restored by peripheral glucocorticoid treatment (Stricker-Krongrad and Beck, 2002). Acute stress, such as immobilization or restrained stress, as well as exposure to cold, increase mRNA levels (Ida et al., 2000; Reyes et al., 2003) and c-fos expression in orexin neurons (Sakamoto et al., 2004; Zhu et al., 2002). The *in vivo* activity of orexin neurons rapidly increases in response to novelty-induced stress (exposure to a novel object in their homecage) and acute immobilization stress (González et al., 2016). An acute stress-induced increase in ACTH levels can be prevented by pretreatment with antagonists for OX1R (Samson et al., 2007) or OX2R (Chang et al., 2007), and is blunted in mice lacking OX2R (Yun et al., 2017) indicating the requirement of both OX1R- and OX2R-mediated signaling for stress response induction.

The use of orexin antagonists in rodents were effective in overcoming the fear response induced by acute footshock stress. A study by Chen et al. investigated fear response (assessed by freezing in rodents) by placing rats in a previously conditioned context. Rats were also placed in a novel context chamber to assess fear to a non-conditioned context (generalized fear). Increased prepro-orexin mRNA levels correlated with the amount of time rats spent freezing in both the shocked and novel context (Chen et al., 2014). The systemic administration of the nonselective dual orexin-A/-B receptor antagonist TCS-1102 was effective in decreasing the freezing response in the shock chamber and inducing anxiolytic effects in a group of rats (termed as high responders) that showed exaggerated freezing to a novel unconditioned tone (Chen et al., 2014). In another study, the anxiolytic effect of orexin receptor antagonism was dependent on the type of stressor (Staples and Cornish, 2014). Rats pretreated with OX1R antagonist SB334867 showed less avoidance from cat odor compared to the vehicle-treated rats. However, another type of stressor, elevated plus maze, did not result in a difference in the behavior of SB334867 versus vehicle-treated rats. It has been suggested that orexin neurons respond to specific forms of stress, likely related with heightened arousal. For example, wakefulness, exploration (associated with arousal) and conditioned fear (associated with both arousal and stress) elicit c-fos expression in orexin neurons. However, restraint stress does not induce c-fos to the same extent in these neurons, suggesting that orexin neurons may be more responsive to specific stressors based on the type of arousal (Furlong et al., 2009).

Orexin has also been found to play a role in anxiety- and panic-like states. The infusion of orexin A into the lateral ventricles of mice had an anxiogenic effect in light-dark box and elevated plus maze paradigms ((Suzuki et al., 2005) but also see (Ito et al., 2008)), which are used to assess anxiety-like behavior. Activation of orexin neurons was necessary to provoke anxiety-like behavior in a rat model of panic

vulnerability induced by the chronic inhibition of GABA synthesis in the DMH-PFA region. Sodium lactate-induced panic response in rats was blocked by the siRNA-mediated downregulation of orexin expression or by systemic treatment with orexin receptor antagonists (Bonaventure et al., 2017; Johnson et al., 2010). CO_2-induced panic-like behaviors and physiological responses were blocked by the selective OX1R antagonist SORA1 (Johnson et al., 2012b) but not by the OX2R antagonist SORA2 (Johnson et al., 2015), suggesting the importance of OX1R signaling for the modulation of panic responses.

The cellular mechanisms underlying the actions of orexin on anxiety-like behavior have also been investigated. Anxiety-like effects of orexin A administration specifically in the BNST assessed by social interaction and elevated plus maze paradigms were dependent on the NMDA receptor function (Lungwitz et al., 2012). Pretreatment with a $GABA_A$ receptor antagonist (bicuculline), an alpha-adrenergic receptor antagonist (phenoxybenzamine) or a beta-adrenergic receptor antagonist (propranolol) blocked orexin A-induced effects on anxiety in the elevated plus maze paradigm (Palotai et al., 2014). These studies suggest that glutamatergic, GABAergic and adrenergic transmission are involved in the orexin-induced anxiogenic effects.

Orexin signaling plays a role in the consolidation and extinction of aversive memories. Orexin has excitatory effects neurons located in the central medial nucleus (Bisetti et al., 2006) which is the major output region of the amygdala and is important for a variety of behaviors, notably (but not limited to) emotional behavior and fear memory. The administration of the OX1R antagonist SB334867 before fear conditioning impairs fear memory when assessed 24 h after training indicating that OX1R is required during the acquisition phase of aversive memories (Sears et al., 2013). SB334867 administration immediately after (but not 4 h after) fear conditioning also reduces fear memory (Flores et al., 2014). These data suggest that OX1R is necessary during early consolidation of fear memories. Fear memory extinction is also affected by modulating the orexin system. Fear extinction is impaired by the icv administration of orexin A, while SB334867 enhances the extinction process (Flores et al., 2014). Accordingly, after establishment of fear memory by fear training, freezing response was assessed by back to back extinction trials for 5 days. Animals were treated with SB334867 after each extinction trial. Contextual freezing was significantly reduced when assessed over each extinction session in animals treated with SB334867 compared to vehicle controls. These effects are dependent on the amygdala but not on the infralimbic prefrontal cortex or hippocampus. In a recent study, orexin neurons were found to be activated during fear extinction, and rats showing poor extinction of cue-induced freezing (high levels of freezing during extinction) had a higher percentage of activated orexin neurons in the hypothalamus (Sharko et al., 2017). This finding suggests that increased activity in orexin neurons may be associated with a greater inability to overcome traumatic experiences.

In summary, acute stress increases orexin mRNA levels and induces orexin neuronal activation. Direct infusion of orexin into the brain leads to stress-related, panic and anxiety-like behaviors. Orexin neurons are also involved in the expression of aversive memories and fear extinction. Orexin receptor antagonists have an anxiolytic effect in response to specific types of stressors. Together, the findings suggest that the orexin system is strongly involved in acute stress responses and that impaired functioning of the orexin system may induce anxiety and panic behavior. Please see Table 1 for the summary of acute stress-induced effects on the orexin system.

3.2. Chronic stress

Although acute stress may be beneficial in situations of threat, prolonged exposure to intense and chronic stress is maladaptive. Chronic stress interferes with the natural physiological coping mechanisms and can cause long-lasting effects on the digestive, immune, cardiovascular and central nervous systems. Prolonged exposure to stress affects the brain by causing alterations in dendritic architecture, synapse density, and neurogenesis. It can also lead to extracellular glutamate-induced excitotoxicity and changes in gene expression (for a review see (McEwen et al., 2015). Repeated stress leads to prolonged HPA activation and increases the risk for depression and anxiety disorders (McEwen, 2008). In addition to alterations of the corticosterone system, dysregulation of the orexin system has been implicated in stress-related disorders. Below, I will review the involvement of the orexin system in animal models of mood disorders induced by different types of chronic stress procedures.

The effects of orexin signaling in chronic restraint stress are dependent on the downstream brain region that is targeted by orexin neurons. Kim et al. demonstrated that the infusion of orexin directly into the basolateral amygdala (BLA) of mice induces depression-like behaviors such as reduced social interaction and increased immobility in forced swim and tail suspension tests that is similar to those seen after chronic restraint stress. The siRNA-mediated suppression of orexin signaling reverses the restraint stress-induced depression-like behaviors (Kim et al., 2015). Conversely, the direct infusion of orexin A into the BNST in mice subjected to chronic restraint stress causes an antidepressant-like behavior in the forced swim test (Chung et al., 2014). Chronic restraint stress also has an inhibitory effect on orexin-induced excitatory presynaptic currents (EPSCs) in layer V pyramidal neurons of the medial prefrontal cortex (mPFC) accompanied by morphological changes in the apical dendrites (Lambe et al., 2007).

Another type of chronic stress paradigm, chronic social defeat stress, leads to decreased prepro-orexin mRNA levels, reduced number of orexin neurons in the LH (Lutter et al., 2008) and decreased orexin levels in the VTA, mPFC and hypothalamus (Nocjar et al., 2012). However, prepro-orexin mRNA levels are lower in the hypothalamus of resilient mice after chronic social defeat stress compared to the levels in susceptible mice (Chung et al., 2014). Interestingly, orexin infusions have different effects in the resilient versus susceptible mice. While orexin A increases social interaction in susceptible mice (indicating an anxiolytic effect) orexinA/B co-treatment does not have an effect. Resilient mice respond to orexinA/B co-treatment with decreased social interaction, but orexin A treatment alone does not show an effect in this group (Chung et al., 2014). Another consequence of chronic social defeat stress is the alterations in the expression levels in OX1R and OX2R mRNAs in the BLA (Arendt et al., 2014). In this model, the shRNA-mediated knockdown of OXR2 in the BLA increases anxiety-like behaviors in social preference and open field tests while OX1R knockdown however does not show an effect on behavior. Further studies using the chronic social defeat stress model are needed to understand the mechanisms underlying the differential effects of orexins in resilient versus susceptible mice and the actions of different orexin receptors on anxiety-like behaviors.

The unpredictable chronic mild stress model (UCMS) in mice has also been used to study the alterations in the orexin system. UCMS-induced increase in c-fos expression in orexin neurons specifically in the dorsomedial-perifornical hypothalamus (DMH-PFA) could be reversed by chronic SSRI fluoxetine treatment in mice. In non-stressed mice, the orexin antagonist almorexant showed an antidepressant-like effect by reducing immobility in the tail suspension test (Nollet et al., 2011). Almorexant was effective in improving UCMS-induced physical and behavioral deficits mimicking the effects of fluoxetine (Nollet et al., 2012). UCMS led to a deterioration in the coat state which reflects an indirect measure of impaired grooming behavior based on a loss of self-care in mice, mimicking a depressive-like state (Yan et al., 2010). UCMS-induced alterations in coat state, weight, behavior and HPA axis were reversed by almorexant. Combination of UCMS with a modified forced swim paradigm resulted in a decrease in orexin A immunoreactive neurons in the LH which was reversed by kosasan with antidepressant-like effects (Ito et al., 2009). The OX1R antagonist SB334867 blocked the antidepressant-like effects of kosasan on behavior. Altogether, these experiments demonstrate that the beneficial

Table 1
Effect of acute stress on the orexin system.

Type of Stress	Alterations in the orexin system	Prevention of stress-induced changes	Reference
Immobilization/restraint	Increased hypothalamic orexin mRNA	Reduction in ACTH levels by OX1R antagonist	Ida et al., 2000 Reyes et al., 2003
	Increased cfos in orexin neurons		Sakamoto et al., 2004 Samson et al., 2007
Cold exposure	Increased hypothalamic orexin mRNA	–	Ida et al., 2000
	Increased cfos in orexin neurons		Sakamoto et al., 2004
Footshock	Increased orexin hypothalamic mRNA	Decrease in freezing and immobility in the open field by OX1R/ OX2R antagonist	Zhu et al., 2002
	Increased hypothalamic prepro-orexin mRNA		Winsky-Sommerer et al., 2004
	Increased cfos in orexin neurons		Chen et al., 2014 Mokhtarpour et al., 2016
Swim stress	Increased cfos in orexin neurons	Reduction in ACTH levels by OX2R antagonist	Chang et al., 2007
Cage exchange	–	Reduction in ACTH levels by OX2R antagonist	Yun et al., 2017
Wakefulness/exploration	Increased cfos in orexin neurons	–	Furlong et al., 2009
Conditioned fear	Increased cfos in orexin neurons	Reduction in fear response by OX1R antagonist or OX1R/OX2R antagonist	Furlong et al., 2009 Steiner et al., 2012 Sears et al., 2013 Flores et al., 2014
Sodium lactate treatment	Increased cfos in orexin neurons	Reduction in panic and cardiovascular response by downregulation of prepro-orexin mRNA	Johnson et al., 2010
		Reduction in anxiety by OX1R antagonist	Bonaventure et al., 2017
CO_2 based normoxic gas infusion	–	Reduction in anxiety and cardiovascular/thermoregulatory response by OX1R antagonist	Johnson et al., 2015

actions of some antidepressant drugs involve alterations in the orexin system.

The orexin system has also a modulatory role on novel stress responses in rodents exposed to prior repeated stress. Orexinergic inputs to the posterior region of the paraventricular nucleus of the thalamus (pPVT) are especially important for adaptive stress responses. Orexin receptor antagonist SB334867 administered into the pPVT prior to repeated swim stress prevents the restraint stress-induced responses in swim-stressed rats. The firing pattern of pPVT cells in response to orexin A and the membrane expression of OX1R is differentially affected in non-stressed and swim-stressed rats. These suggest that altered orexin signaling in pPVT neurons may underlie the ability to adapt to chronic stress (Heydendael et al., 2011).

Early maternal separation is often used as a model of chronic stress and causes characteristic stress-related effects in adulthood (Vetulani, 2013). Maternal separation in neonatal rats causes sleep disturbances that are accompanied by an increase in CRH and orexin A levels in the hypothalamus of adult rats. Alterations in orexin B levels and the protein expression of OX1R and OX2Rs in the projection sites including the hippocampus and frontal cortex were also observed using this model (Feng et al., 2007). Rats previously subjected to maternal separation had decreased c-fos expression in orexin neurons after restraint stress. The control rats which were not subjected to early life stress had enhanced c-fos expression in orexin neurons after restraint stress. This suggested that early life stress causes a hypoactive orexin system in rats when they are subjected to psychological stress in adulthood (James et al., 2014).

Alterations in the number of orexin neurons were found in animal models with a depressive-like phenotype. A detailed immunohistochemical and stereological analysis in WKY rats with depression-like characteristics (Will et al., 2003) revealed a significant decrease in orexin A-positive neurons and a reduction in orexin A neuronal soma size when compared with the controls (Allard et al., 2004). In contrast, Flinders Sensitive Line rats, which also show depressive-like behavior and sleep disturbance, had an increased number of orexin neurons compared to their Flinders Resistant Line control strain (Mikrouli et al., 2011). Increased orexin levels in hypothalamus was detected by the neonatal administration of the antidepressant clomipramine which induces depressive-like behavior in adult rats (Feng et al., 2008) and with the corticosterone injection-induced depression model in rats (Jalewa et al., 2014). These studies further strengthened the link between the impairments in the orexin system and depression-like behaviors.

The effects of chronic stress on the orexin system are variable depending on the type of the stressor and the brain region involved. In general, chronic stress leads to changes in the number of orexin neurons, orexin mRNA levels and orexin neuronal activation. While orexin receptor antagonism produces an antidepressant-like effect in certain chronic stress paradigms, it increases anxiety-like behaviors in others. Orexin infusion into one brain region produces an antidepressant-like behavior, while in another region, it increases depressive-like behavior. Orexin infusion also has differential effects in rodents that respond differently to chronic stress. As orexin neurons project to and regulate a wide variety of downstream projection regions involved in stress and anxiety-like behaviors, this variability is not surprising. The emergence of novel techniques that allow the manipulation of specific orexin neuronal activity *in vivo* holds promise for uncovering the circuit-specific modulatory effects of orexin on the stress response. Please see Table 2 for the summary of chronic stress-induced effects on the orexin system.

4. Optogenetic and chemogenetic studies on the orexin system

4.1. Sleep/wakefulness

Heightened arousal is a key component of the stress response. Disturbances in sleep and arousability are among the key symptoms associated with stress-related psychiatric diseases (Benca, 1996; Krystal, 2012). For example, fragmented sleep, frequent awakenings, decreased sleep time and nightmares are commonly observed in patients with PTSD (van Liempt et al., 2006). Interestingly, chronic stress-induced startled awakenings in rats similar to those seen in PTSD were associated with decreased orexin activity (Yu et al., 2016). Given the important role of orexin in arousability, the first *in vivo* optogenetic studies on orexin's role in sleep and wakefulness.

Adamantidis et al. injected the LH of mice with a lentivirus encoding the light-activated cation channel channelrhodopsin2 and mCherry (ChR2-mCherry) protein under the control of the prepro-orexin promoter (Adamantidis et al., 2007). This enabled the orexin neurons to be specifically activated using blue light. The *in vivo*

Table 2
Effect of chronic stress on the orexin system.

Type of Stress	Alterations in the orexin system	Prevention of stress-induced changes	Reference
Chronic restraint	Decreased EPSCs in pyramidal neurons of mPFC		Lambe et al., 2007
Chronic social defeat	Decreased prepro-orexin mRNA	Increased social interaction in susceptible mice by orexin A infusion	Lutter et al., 2008
	Reduced number of orexin neurons		Nocjar et al., 2012
	Decreased orexin levels in hypothalamus, VTA and mPFC		Arendt et al., 2014
	Increased OX1R mRNA and decreased in OX2R mRNA in BLA		Chung et al., 2014
Unpredictable chronic mild stress	Increased cfos in orexin neurons	Improved coat state, reduced weight gain, decreased immobility in TST, decreased anxiety in EPM by OX1R/OX2R antagonist	Nollet et al., 2011 Nollet et al., 2012
Chronic mild stress followed by swim stress	Decreased orexin A immunoreactive neurons		Ito et al., 2009
Repeated swim stress followed by restraint	Increased OX1R internalization in pPVT	Decreased anxiety in EPM by OX1R antagonist	Heydendael et al., 2011
Early maternal separation	Increased orexin A in hypothalamus		Feng et al., 2007
	Reduced orexin B in hippocampus		James et al., 2014
	Altered OXR expression in projection sites		
	Restraint-induced cfos in orexin cells is dampened		
Wistar-Kyoto rats	Decreased prepro-orexin mRNA, orexin A immunoreactive neurons and orexin neuronal soma size		Taheri et al., 2001 Allard et al., 2004
Flinders Sensitive Line rats	Increased number of orexin neurons		Mikrouli et al., 2011
Neonatal clomipramine	Decreased orexin levels in multiple brain regions in juvenile rats		Feng et al., 2008
	Increased hypothalamic orexin levels in adult rats		
Corticosterone treatment	Increased orexin expression in hypothalamus		Jalewa et al., 2014

photostimulation of orexin neurons significantly reduced the latency to wakefulness without affecting the total wake or sleep durations. Stimulation of orexin neurons was sufficient to enhance the probability of an awakening event throughout the entire light/dark period (Carter et al., 2009). Inhibition of noradrenergic neurons of the LC during orexin neuron stimulation blocked orexin-mediated effects on sleep to wake transitions, suggesting that the LH-LC interaction is critical for the role of orexin signaling in arousal (Carter et al., 2012).

To optogenetically silence orexin neurons, Tsunematsu et al. generated transgenic mice that express the chloride pump halorhodopsin under the control of the orexin promoter. In these mice, stimulation of halorhodopsin by orange light hyperpolarized the membrane potential and inhibited action potentials in orexin neurons (Tsunematsu et al., 2011). Silencing orexin neurons during the light period (inactive period) induced slow wave sleep, while a longer photoinhibition was required to increase slow wave sleep during the dark period.

Other studies utilized a chemogenetic approach to manipulate neuronal activity based on the use of Designer Receptors Exclusively Activated by Designer Drugs (DREADDs), which are extrinsic muscarinic receptors that can be activated by a synthetic ligand clozapine-*N*-oxide (CNO). The expression of cre-dependent AAV viruses containing DREADDs in the LH of orexin-cre transgenic mice allowed *in vivo* manipulation of orexin neuronal activity upon CNO administration (Sasaki et al., 2011). They showed that activation of orexin neurons was associated with increased awake time and decreased sleep time while inhibition of orexin neurons led to decreased awake time accompanied by increased sleep time. Although there have been recent concerns regarding the specificity of CNO (Gomez et al., 2017), these initial *in vivo* studies successfully manipulated the activity of orexin neurons. This strengthened the perceived role of the orexin system in the maintenance of arousal states.

4.2. Emotional memory

Optogenetic studies have investigated the fear memory circuits controlled by orexin. Stimulation of orexin terminals in the LC in conjunction with the tone-shock pairing during fear conditioning enhanced long-term fear memory (Sears et al., 2013). Optogenetic inhibition of orexin fibers in the LC after cued fear conditioning reduced cued fear memory. Optogenetic activation of orexinergic fibers in the LC or the noradrenergic fibers in the LA during the fear memory test in the absence of the auditory cue induced robust freezing in a novel context, indicating fear generalization (Soya et al., 2017). These experiments suggested that the LA-LC-amygdala circuit is important both for fear memory expression and for fear generalization. Generalization of fear memories is often associated with conditions such as panic disorders or PTSD indicating a role for the orexin system in these diseases.

4.3. Stress

Stimulation or inhibition of orexin neurons optogenetically or chemogenetically can modulate the regulation of stress responses. Heydendael et al. investigated the effects of optogenetic stimulation of orexin neurons on anxiety in the social interaction test. Rats transduced with a ChR2-YFP virus spent significantly less time engaged in social interaction compared to rats expressing the control YFP virus after photostimulation (Heydendael et al., 2014). Interaction with a novel rat is often curtailed by anxious individuals. Total distance traveled in the social interaction test arena was increased by photostimulation; however, distance traveled in the homecage remained similar, indicating increased arousal or locomotion in a novel context. In response to phasic high frequency optogenetic stimulation of orexin neurons in the LH, physiological parameters linked to the stress response, including plasma corticosterone levels, heart rate, and blood pressure, were elevated and open field exploratory behavior was disrupted (Bonnavion et al., 2015). An orexin-dependent increase in corticosterone concentrations is regulated in part by the satiety hormone leptin, which suppressed stimulation-induced orexin cell activity and corticosterone release (Bonnavion et al., 2015). An association between orexin neuron activation and a negative emotional valence became stronger with the studies showing that orexin neuronal activation paired with one side of a chamber is sufficient to make the mice move away from that side of the chamber (Giardino et al., 2018). Together, these studies show that optogenetic stimulation of orexin neurons leads to anxiety-like behavior, increases the HPA axis activity and initiates a generalized stress response.

Several studies used DREADDs to investigate orexin effects in stress. Campbell et al. showed that expression of the artificial excitatory receptor hM3Dq followed by administration of its ligand CNO in LH induced c-fos activity in LH cells including but not limited to orexin neurons. Chemogenetic activation of LH neurons reversed early life stress-induced deficits in a motivated response for sucrose in rats (Campbell et al., 2017). The orexin system was also implicated in the sex differences to chronic stress. Female rats showed elevated orexin activity as well as reduced habituation, impaired cognitive flexibility and a heightened HPA axis response in response to repeated restraint stress when compared with male rats (Grafe et al., 2017a). Expression of the artificial inhibitory receptor, hM4Di specifically in orexin neurons followed by CNO administration during repeated restraint stress improved habituation to stress, restored the elevated HPA axis responses and improved cognitive flexibility in female rats. In male rats, stimulation of hM3Dq-expressing orexin neurons by CNO prior to each restraint increased struggle behavior, as assessed by evaluating the number of escape attempts during the stress procedure (Grafe et al., 2017b). The resilience to social defeat stress on the other hand, was associated with lower prepro-orexin mRNA levels. Chemogenetic inhibition of orexin activity during stress reversed the effects of social defeat and promoted resilience in the previously susceptible rats (Grafe et al., 2018). These experiments suggest that manipulation of orexin activity has beneficial outcome on chronic stress-induced impairments however, the direction of the manipulation may be dependent on the sex and the type of stress.

Recent studies investigated the orexin-mediated circuitries in stress- and anxiety-related behaviors using optogenetic and chemogenetic approaches. Excitatory orexin projections onto glutamic acid decarboxylase 65 (GAD65) cells within the LH were found to be critical for stress and locomotion (Kosse et al., 2017). Immobilization stress-induced rapid activation in the LH GAD65 neurons was dependent on the OX1R receptor. Chemogenetic inhibition of LH GAD65 neurons reduced voluntary locomotion in mice, whereas activation of LH GAD65 neurons increased this locomotion. Therefore, orexin neurons act as upstream regulators of GAD65 cellular activity, which increases in response to stress and locomotion. Orexin neurons were also found to enhance risk-avoidance behavior, which may be associated with increased anxiety. Suppression of orexin signaling as well as chemogenetic inhibition of D2 expressing medium spiny neurons in the NAc inhibited risk avoidance suggesting that inhibition of risk-taking behavior is dependent on orexin-induced activation of NAc D2 neurons (Blomeley et al., 2018).

Recent optogenetic and chemogenetic studies have strengthened the role of the orexin system in sleep and arousal. Increased orexin neuronal activity was associated with increased awake time and decreased sleep time. Inhibition of orexin neurons on the other hand, decreased wakefulness and induced sleep. Recent studies also showed that orexin release in the LC is important for fear memory. Inhibition of orexin fibers in the LC decreased fear memory while stimulation of orexin fibers in the LC led to enhanced fear. Follow up studies demonstrated the effects of orexin neuronal activation and inhibition on stress and anxiety. Acute optogenetic stimulation of orexin neurons decreased social interaction time, increased locomotion in a novel context and enhanced the physiological stress responses. Inhibition of orexin neurons in female rats improved chronic-stress induced changes in stress habituation, HPA axis response, and cognitive flexibility. Orexin neuron inhibition in male rats during stress reversed the stress-induced changes in social behavior. Lastly, orexin neurons were found to act as upstream regulators of different sets of neurons which are involved in stress, locomotion and innate risk-avoidance behaviors. Please see Table 3 for the summary of optogenetic and chemogenetic studies on the orexin system and the resulting behavioral and physiological findings.

5. Orexin in depression and anxiety disorders

Orexin has been implicated in human depression and anxiety disorders in a number of studies. Deficiency in orexin has long been known to cause narcolepsy in humans (Nishino et al., 2000; Peyron et al., 2000; Thannickal et al., 2000), and narcolepsy is strongly associated with depression (Daniels et al., 2001). CSF orexin levels were significantly lower in patients with major depressive disorder (MDD) (Salomon et al., 2003), in depressed patients with suicidal tendencies (Brundin et al., 2007) and in PTSD patients (Strawn et al., 2010). In another study, higher orexin levels were detected in the CSF of patients with panic anxiety (Johnson et al., 2010). In healthy subjects, positive emotions such as laughter or excitement, correlated with high orexin levels in the amygdala, whereas reduced orexin levels were found during periods associated with sadness or frustration (Blouin et al., 2013). These studies indicate that orexin levels are altered in depression and anxiety disorders and are highly correlated with the emotional state of the individuals.

Several follow up studies identified differential results with respect to orexin and orexin receptor expression in the hypothalamus of patients with MDD. Postmortem analysis revealed increased hypothalamic orexin, impaired diurnal fluctuation in OX1R levels and increased OX2R mRNA expression in the anterior cingulate cortex of depressed patients (Lu et al., 2017). Several other studies reported no changes in the expression levels of orexin in patients with MDD. Rotter et al. detected methylation differences in the promoter region of the *orexin A* gene that may cause differences in the expression levels. However, the detected decrease in orexin A mRNA expression did not reach significance (Rotter et al., 2011). A negative correlation between orexin A levels and symptom severity was evident. In addition to the lack of changes in orexin levels, a lack of correlation between orexin levels and symptom severity in MDD was also reported in some studies in which a relatively small number of subjects was included (Schmidt et al., 2011, 2010). The heterogeneity of depressive disorders as well as differences in the study design and methodological limitations may account for these conflicting results.

A detailed genotyping single nucleotide polymorphism (SNP) analysis in patients with major mood disorders for the *Hcrt, Hcrtr1* and *Hcrtr2* genes revealed that a polymorphism in the *Hcrtr1* gene was significantly associated with depression. The A allele of this polymorphism was found to increase the disease risk by 2-fold (Rainero et al., 2011). The rs2271933 G > A polymorphism led to the substitution of a valine residue for isoleucine (Ile408Val) in the amino acid sequence of the OX1R. In another study the Iso allele of the Val308Iso polymorphism in the *Hcrtr2* gene (Val308 Iso polymorphism) was more frequent in female patients with panic disorder compared to control subjects (Annerbrink et al., 2011). These studies suggest that orexin receptor protein function may be compromised in depressive and panic disorders; however, the mechanisms underlying the functional effects of these polymorphisms remain to be elucidated.

In summary, the majority of the studies in depressed/PTSD patients reported a decrease in CSF orexin levels and/or alterations in the diurnal variation of orexins. However, patients with panic disorder had increased CSF orexin levels. Postmortem tissue of depressed patients had an increased mRNA expression levels of orexin and orexin receptor. Studies have also identified polymorphisms in the genes encoding the orexin receptors. Taken together, these studies show that targeting the orexin system in different psychiatric disorders may have therapeutic potential for these diseases.

6. Conclusion

Orexins have been one of the most widely studied peptides due to their role in regulating diverse physiological functions. Because orexin neurons innervate a wide variety of brain regions that are also implicated in stress and emotional modulation, it is not surprising that

Table 3
In vivo manipulation of orexin activity and changes in behavioral and physiological functions.

Model	Manipulation	Behavior	Reference
ChR2 expression in orexin neurons	Activation of orexin neurons	Reduced latency to awake state from SWS and REM sleep	Adamantidis et al., 2007
		Increased probability of sleep to wake transitions	Carter et al., 2009
		Increased probability of an awakening event during light/dark period	Rolls et al., 2011
		Induced sleep fragmentation	Heydendael et al., 2014
		Impaired novel object recognition memory	Bonnavion et al., 2015
		Increased social anxiety	Giardino et al., 2018
		Increased locomotion in a novel environment	
		Decreased exploratory behavior in the open field	
		Increased corticosterone levels, heart rate, blood pressure	
		Behavioral avoidance	
hM3Dq expression in orexin neurons	Activation of orexin neurons	Increased awake time	Sasaki et al., 2011
		Decreased non-REM and REM sleep times during light/dark period	Grafe et al., 2017a,b
		Increased struggle behavior during stress	
ChR2 expression in orexin neurons	Activation of orexin terminals in LC	Enhanced fear memory	Sears et al., 2013
		Fear generalization	Soya et al., 2017
Halorhodopsin expression in orexin neurons	Inhibition of orexin neurons	Increased SWS during the light period	Tsunematsuet al., 2011
Archaerhodopsin T expression in orexin neurons	Inhibition of orexin neurons	Increased SWS during the dark period	Tsunematsu et al., 2013
hM4Di expression in orexin neurons	Inhibition of orexin neurons	Decreased awake time	Sasaki et al., 2011
		Increased non-REM sleep time during light/dark period	Grafe et al., 2018
		Improved habituation to restraint stress	Grafe et al., 2017b
		Decreased stress-induced HPA axis response	
		Improved cognitive flexibility after stress	
		Increased social interaction after stress	
		Decreased immobility in FST after stress	
Long-wavelength vertebral cone opsin expression in orexin neurons	Inhibition of orexin terminals in LC	Decreased fear memory	Soya et al., 2017

orexins are critical for stress response regulation. Activation of orexin neurons is associated with acute stress responses. However, the effects of chronic stress on the orexin system are variable. Dysfunction of the orexin system has been implicated in depression and anxiety disorders, but the mechanisms are unclear and the results are not always consistent. Recent technology allowing the identification of the circuit-specific neuromodulatory effects of orexin on the stress response will advance our understanding of orexin's role in stress regulation and may pave the way for novel treatments that target the orexin system to treat mental health disorders.

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