A. Shekhar (Ed.)
Progress in Brain Research, Vol. 198
ISSN: 0079-6123

CHAPTER 9

Orexin, stress, and anxiety/panic states

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Abstract: A panic response is an adaptive response to deal with an imminent threat and consists of an integrated pattern of behavioral (aggression, fleeing, or freezing) and increased cardiorespiratory and endocrine responses that are highly conserved across vertebrate species. In the 1920s and 1940s, Philip Bard and Walter Hess, respectively, determined that the posterior regions of the hypothalamus are critical for a "fight-or-flight" reaction to deal with an imminent threat. Since the 1940s it was determined that the posterior hypothalamic panic area was located dorsal (perifornical hypothalamus: PeF) and dorsomedial (dorsomedial hypothalamus: DMH) to the fornix. This area is also critical for regulating circadian rhythms and in 1998, a novel wake-promoting neuropeptide called orexin (ORX)/hypocretin was discovered and determined to be almost exclusively synthesized in the DMH/PeF perifornical hypothalamus and adjacent lateral hypothalamus. The most proximally emergent role of ORX is in regulation of wakefulness through interactions with efferent systems that mediate arousal and energy homeostasis. A hypoactive ORX system is also linked to narcolepsy. However, ORX role in more complex emotional responses is emerging in more recent studies where ORX is linked to depression and anxiety states. Here, we review data that demonstrates ORX ability to mobilize a coordinated adaptive panic/defense response (anxiety, cardiorespiratory, and endocrine components), and summarize the evidence that supports a hyperactive ORX system being linked to pathological panic and anxiety states.

Keywords: hypocretin; hypothalamus; arousal; panic; anxiety; circadian; perifornical; GABA; glutamate; diurnal.

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http://dx.doi.org/10.1016/B978-0-444-59489-1.00009-4

Orexin/hypocretin discovery and loss of function linked to narcolepsy

Orexins (ORXs) are hypothalamic neuropeptides that were simultaneously discovered in 1998 by two different research groups (de Lecea et al., 1998; Sakurai et al., 1998). They determined that there are two forms of ORXs, ORX-A and ORX-B (also, respectively, known as hypocretin 1 and 2), that are produced from a common prepro-ORX precursor that are endogenous ligands for the G-protein-coupled ORX1 and ORX2 receptors (see inset in Fig. 1a). The ORX1 receptor has greater affinity for ORX-A than ORX-B, whereas the ORX2 receptor has similar affinity for both ORX-A and ORX-B (Sakurai et al., 1998; see also Chapter 2 of this volume).

Yet, the most striking part of this discovery was that, within the rat brain, the distribution of the ORX-synthesizing neurons was restricted primarily to the specific subnuclei in posterior regions of the hypothalamus that are dorsal, medial, and lateral to the fornix (Peyron et al., 1998), with their terminals reaching almost every part of the CNS. For decades prior to the discovery of the neurochemical phenotype of these neurons, these hypothalamic neurons have been shown to regulate a wide range of behavioral and physiological responses such as circadian rhythms (e.g., dorsomedial region; Chou et al., 2003; Gooley et al., 2006), anxiety and cardiorespiratory regulation (perifornical and dorsomedial region; DiMicco et al., 2002; McDowall et al., 2006; Shekhar and DiMicco, 1987; Shekhar and Katner, 1995), and feeding and reward (lateral region; Gutierrez et al., 2011).

In the initial study by Sakurai and colleagues, ORX was shown to have mild effects on food intake which led to the name "orexin" (Sakurai et al., 1998). Yet very soon after ORX discovery, loss of ORX function was strongly linked to narcolepsy, which is a sleep disorder that is associated with sudden and brief episodes of sleep and cataplexy that can be triggered by strong emotions (Peyron et al., 2000; Thannickal et al., 2000). Preclinical studies showed that ORX knockout mice displayed a narcoleptic-like sleep disruption (based on behavior and EEG activity) (Chemelli et al., 1999), and that a mutation in the ORX2 receptor in the Doberman was the cause of hereditary narcolepsy (Lin et al., 1999). Clinical studies later determined that central levels of ORX (Mignot et al., 2002; Nishino et al., 2000) and number of ORX neurons (Peyron et al., 2000; Thannickal et al., 2000) are dramatically reduced in humans with narcolepsy. ORX was later determined to be colocalized with glutamate (Henny et al., 2010; Torrealba et al., 2003) and also dynorphin (Chou et al., 2001). Therefore, in humans, the narcolepsy condition, which is associated with loss of ORX neurons, may also reflect loss of colocalized glutamate and the neuropeptide dynorphin.

Further studies reinforced ORX role in promoting arousal and wakefulness (Adamantidis et al., 2007; Chemelli et al., 1999; Hara et al., 2001; see Chapter 3) and regulating energy balance and reward (see reviews Boutrel et al., 2010; Harris and Aston-Jones, 2006; see Chapters 5–7). Consistent with that role, *in vivo* electrophysiological recordings of ORX neurons reveal that ORX neuronal activity is higher during wake periods, compared to periods of sleep. In that study, they also noted that ORX neuronal activity is higher during behavior that requires risk assessment (i.e., exploration), rather than appetitive behavior that occurs in the absence of threat (i.e., feeding or grooming) (Mileykovskiy et al., 2005). Overall, this suggested that increases in ORX activity beyond what is needed for wake maintenance may be associated with increased vigilance, a trait associated with anxiety states. Consistent with this hypothesis, initial studies began to demonstrate that ORX also regulates a variety of emotional (Kayaba et al., 2003), endocrine (Al-Barazanji et al., 2001; Russell et al., 2001; Samson et al., 2002), cardiovascular (Chen et al., 2000; Ciriello et al., 2003; Machado et al., 2002; Samson et al., 1999), and respiratory (Kayaba et al., 2003) responses associated with an integrative stress response. Here, we will

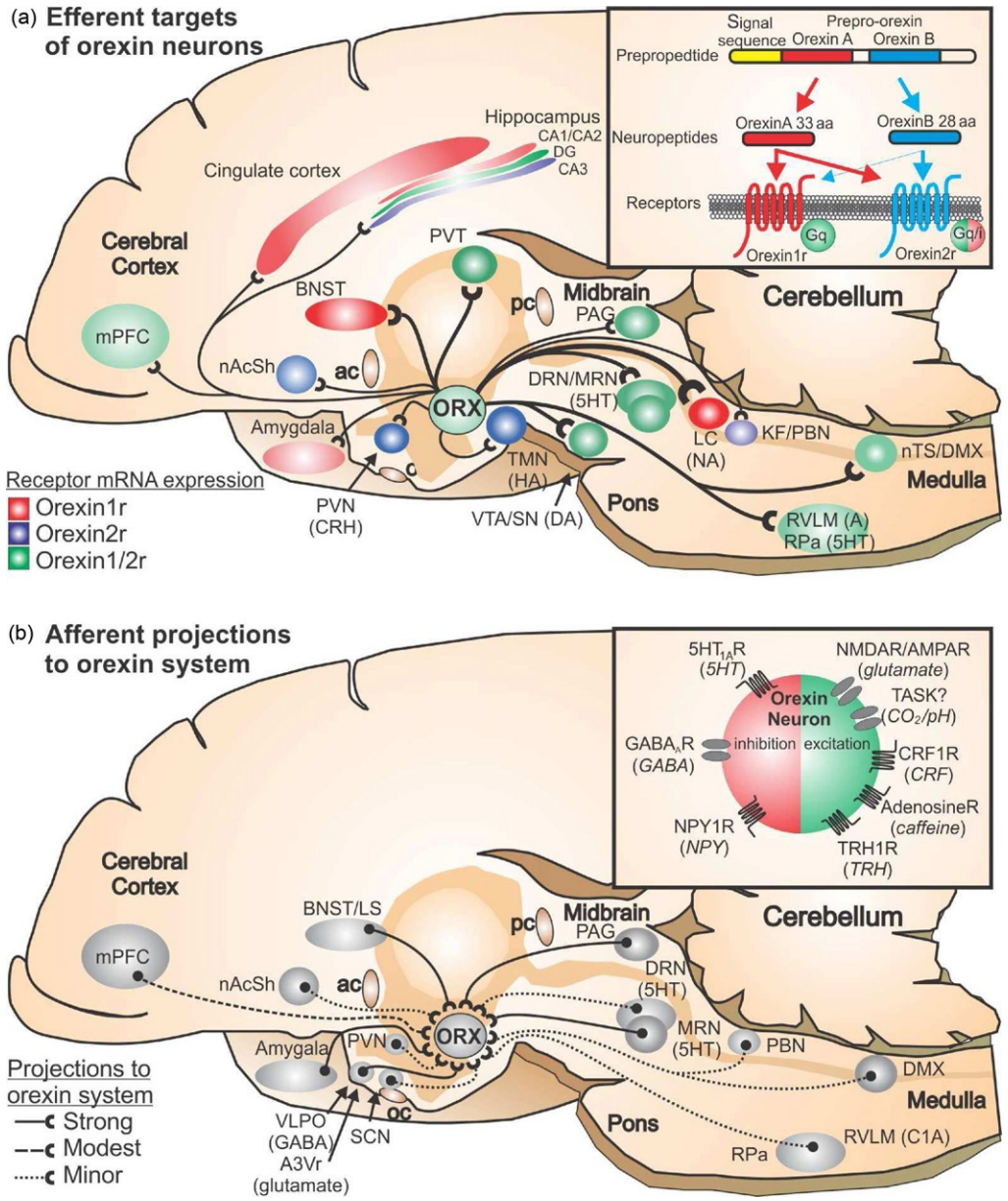

Fig. 1. (a) Efferent targets of orexin neurons—a midsagittal section of a rat brain illustrating the density of orexin projections to postsynaptic targets sites (Nambu et al., 1999; Peyron et al., 1998) and density of the orexin 1 and/or 2 receptor at these projection sites (Marcus et al., 2001; Trivedi et al., 1998). The density of the orexin projections is represented by the line thickness and the density of expression of orexin 1 and 2 receptor mRNA is, respectively, represented by the intensity of the associated color; (b) afferent projections to orexin neuronal system—a midsagittal section of a rat brain illustrating the density of brain regions that project onto orexin neurons (Sakurai et al., 2005; Yoshida et al., 2006). Density is indicated by solid or type of dashed line at the legend at bottom right of the illustration. 5HT, serotonergic; A, adrenergic; A3V, anterior third ventricle

review anatomical and functional studies that demonstrate that ORX system is located in a well-established aversive motivation (Shekhar et al., 1987) and panic-generating site (i.e., perifornical hypothalamus (PeF) and dorsomedial hypothalamus (DMH); Hess, 1954; Hess and Akert, 1955; Shekhar and DiMicco, 1987; Shekhar et al., 1990) and is a critical substrate for an adaptive panic response in the presence of a threat (either external or internal). Further, here we will review recent data linking a hyperactive ORX system to anxiety pathology associated with panic disorder (Johnson et al., 2010b).

Neuroanatomical evidence supporting a role for orexin/hypocretin involvement in anxiety and panic

Neuroanatomical connections of the orexin system are consistent with a role in anxiety and panic

Efferent targets of orexin neurons

Although ORX neuronal projections are present throughout the brain, they are particularly dense in areas of the brain that mobilize different components of a panic response (Nambu et al., 1999; Peyron et al., 1998) such as the (1) *stress and arousal systems*—medial prefrontal cortex (mPFC; Gabbott et al., 2005; Kim and Whalen, 2009), cingulate cortex and monoaminergic systems (e.g., noradrenergic locus ceruleus (LC; Itoi and Sugimoto, 2010), serotonergic dorsal raphe nucleus (DRN; Lowry et al., 2005)), and histaminergic tuberomammillary hypothalamic nucleus (TMN)); (2) *anxiety and panic emotion centers*—limbic brain regions (e.g., bed nucleus of the stria terminalis (BNST; Duvarci et al., 2009; Fox et al., 2010; Lee et al., 2008; Sahuque et al., 2006; Sajdyk et al., 2008a), lateral septum (LS; Bakshi et al., 2007; Henry et al., 2006), and central amygdala (CeA; Rainnie et al., 2004; Sajdyk et al., 2008b; Shekhar et al., 2005; Lungwitz et al., 2012; Tye et al., 2011)); (3) *autonomic sites*—adrenergic rostroventrolateral medulla (RVLM), serotonergic raphe pallidus (RPa), periaqueductal gray (PAG), nucleus of the solitary tract (nTS), and dorsal motor nucleus of the vagus (DMX) (see reviews Dampney et al., 2005; McDowall et al., 2006); (4) *respiratory sites*—parabrachial nucleus (PBN), Kölliker-Fuse nucleus (KF), retrotrapezoid nucleus (RTN),and pre-Bötzinger complex (Pre-Botz) (see review Guyenet et al., 2010; Chapter 4); and (5) *stress hormone sites*—paraventricular hypothalamus (PVN) for stress hormone release and sympathetic activation (Swanson et al., 1983). Other areas of dense innervation occur in motivated behavior-related brain regions such as the ventral tegmental area and nucleus accumbens shell.

In many regions of the brain, the expression of ORX1 and ORX2 receptors is coexpressed (Marcus et al., 2001; Trivedi et al., 1998). Yet many other areas have fairly selective expression of the ORX2 or ORX1 receptors. For instance, the ORX2 receptor is almost the exclusive ORX receptor in the histaminergic neurons in the TMN which plays a critical role in wake promotion (Bayer et al., 2001; Huang et al., 2001); PVN neurons which express corticotropin-releasing hormone (CRH) to initiate the hypothalamic–pituitary–adrenal (HPA) axis hormone cascade (Vale et al., 1981); and the PBN which regulates breathing (Hayward et al.,

region; ac, anterior commissure; BNST, bed nucleus of the stria terminalis; CRH, corticotropin-releasing hormone; DA, dopaminergic; DMX, dorsal motor nucleus of the vagus; DRN, dorsal raphe nucleus; HA, histaminergic; KF, Kölliker-Fuse nucleus; LC, locus ceruleus; LS, lateral septum; mPFC, medial prefrontal cortex; MRN, median raphe nucleus; NA, noradrenergic; nAcSh, nucleus accumbens shell; nTS, nucleus of the solitary tract; oc, optic chiasm; PAG, periaqueductal gray; pc, posterior commissure; PBN, parabrachial nucleus; PVN, paraventricular hypothalamic nucleus; PVT, paraventricular thalamus; RPa, raphe pallidus; RVLM, rostroventrolateral medulla; SCN, suprachiasmatic nucleus; SN, substantia nigra; TMN, tuberomammillary nucleus; VLPO, ventrolateral preoptic area; VTA, ventral tegmental area.

2004). Conversely, ORX1 receptors are selective for the limbic system (BNST and amygdala), cingulate cortex, and noradrenergic LC. The significance of the receptor expression patterns as it relates to anxiety and panic responses is discussed in subsequent sections.

ORX release is also excitatory at many of these efferent targets of the ORX neurons that are associated with stress responses. For instance, ORX excites noradrenergic neurons in the LC and increases arousal (Hagan et al., 1999; Horvath et al., 1999). Further, ORX infusion into the LC increases norepinephrine (NE) release at efferent targets of the LC noradrenergic system that are associated with arousal (i.e., dentate gyrus) (Walling et al., 2004). ORX also excites serotonergic neurons in the DRN (Brown et al., 2002) and adrenergic neurons in the RVLM *in vitro* (Antunes et al., 2001). This further supports ORX involvement in anxiety since many anxiolytic compounds target monoaminergic systems generally using tricyclic antidepressants (Rifkin et al., 1981) or monoamine oxidase inhibitors (Kelly et al., 1971) or by using drugs that alter activity of specific monoaminergic systems (e.g., serotonergic or norepinephrine reuptake inhibitors; see review Cloos and Ferreira, 2009).

Afferent projections to the orexin system

In 2005, Sakurai and colleagues genetically encoded a retrograde tracer in ORX neurons to determine afferent systems that made synaptic contacts with ORX neurons (Sakurai et al., 2005). This study revealed a number of brain regions with prominent projections onto ORX neurons, which included limbic brain regions (e.g., BNST, LS, and medial amygdala), many hypothalamic nuclei (e.g., PVN and GABAergic neurons in the ventrolateral preoptic area), serotonergic neurons in the midbrain median raphe nucleus, C1 adrenergic neurons in the RVLM, and neurons in the DMX. In 2006, Yoshida and colleagues identified prominent afferent systems to the ORX system using traditional retrograde tracing (Yoshida et al., 2006). Once identified, they then injected anterograde tracers into the most prominent afferent system and looked for appositions on ORX neurons. In addition to confirming many afferent systems identified by Sakurai and colleagues, they also found robust projections from the mPFC, CeA, PAG, and DRN.

Overall, these neuroanatomical data suggest that ORX neurons are ideally interconnected with known anxiety and panic brain regions to integrate a variety of stress-associated sensory signals (e.g., external threats associated with vision, smell, and hearing, or internal threats associated with autonomic tone, respiratory patterns, and plasma parameters such as hormone, salt, or PCO_2/pH levels) and mobilize adaptive behavioral and physiological response to deal with the threat and restore homeostasis.

Anxiety- and panic-related neurochemical input onto ORX neurons

Many neurochemical systems (many of which arise from the previous section on afferent projections to the orexin system: e.g., serotonergic, noradrenergic, adrenergic, corticotropin-releasing factor (CRF), and GABAergic) regulate ORX neuronal activity (see inset in Fig. 2b). ORX-producing neurons contain $GABA_A$ receptor subunits (Backberg et al., 2002) and are inhibited by the $GABA_A$ receptor agonist muscimol (Eggermann et al., 2003) and excited by the $GABA_A$ receptor antagonist bicuculline methiodide (BMI; Alam et al., 2005). This is notable since local disinhibition of the ORX neuron containing medial hypothalamic regions with BMI evokes strong panic-associated responses in rats, which is discussed in section "The PeF role in adaptive panic/"fight-or-flight" responses". Glutamate also excites ORX neurons via AMPA and NMDA receptors (Li et al., 2002). As stated in section "Efferent targets of orexin neurons", ORX excites both noradrenergic and serotonergic neurons in the

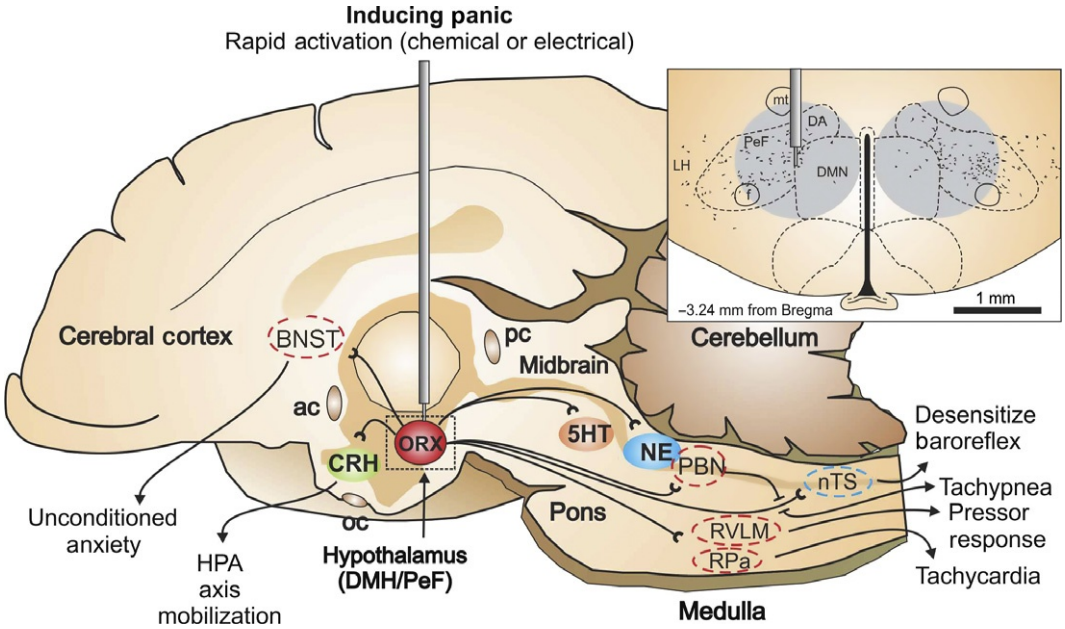

Fig. 2. Illustrates a midsagittal image of the rat brain showing the neural circuits that mobilize panic-associated behavior and cardiorespiratory responses following rapid loss of GABA inhibition with a GABAA receptor antagonist (i.e., bicuculline methiodide), or chronic infusion of a GABA synthesis inhibitor (i.e., L-allylglycine) followed by an intravenous sodium lactate infusion. The top right panel illustrates a coronal hypothalamic section with subnuclei of the hypothalamus delineated by dashed lines with reference to a standard stereotaxic atlas of the rat brain. The perifornical hypothalamus (PeF) is just dorsal to the fornix (f), and the dorsomedial hypothalamus (DMH) collectively contains the dorsal hypothalamic area (DA), and the dorsomedial hypothalamic nucleus (DMN). The gray circle in the panel indicates the target sites for injections/infusions and estimated diffusion distances. The orexin neurons are indicated by solid black traces from a rat brain section immunostained for orexin A. The vast majority of these neurons are located in the perifornical nucleus (PeF), with the remainder mainly located in the lateral and dorsomedial hypothalamic nuclei (LH and DMH). 3V, 3rd ventricle; ac, anterior commissure; BNST, bed nucleus of the stria terminalis; f, fornix; mt, mammillothalamic tract; nTS, nucleus of the solitary tract; pc, posterior commissure; PBN, parabrachial nucleus; RPa, raphe pallidus; RVLM, rostroventrolateral medulla.

midbrain/pons. Subsequent postsynaptic release of NE and 5-HT onto ORX neurons may reflect negative feedback since ORX neurons are directly inhibited by 5-HT (5-HT_{1A} receptor-mediated), and indirectly inhibited by NE through its effects on presynaptic α2 adrenergic receptors on glutamatergic terminals in contact with ORX neurons (effectively inhibiting glutamate release) (Li et al., 2002; Yamanaka et al., 2006). NE also has direct effects on ORX neurons via α1 adrenergic neurons, but this is weak in comparison to the indirect inhibitory effects (Yamanaka et al., 2006).

There is evidence suggesting that the ORX neurons may be a critical target of CRF neurons that are heavily implicated in the mobilization of vigilance behavior and an integrative stress response. In addition to endocrine functions associated with mobilization of the HPA axis (in this instance synthesized in the PVN and referred to as CRH; Vale et al., 1981), the neuropeptide CRF is synthesized (and released) in other extrahypothalamic brain regions (Keegan et al., 1994; Swanson and Simmons, 1989), where it acts as a neurotransmitter/neuromodulator to coordinate

behavioral and autonomic responses to stress. For instance, central injections of CRF mobilize "panic/defense" responses in rodents (Brown et al., 1988; Ku et al., 1998). The CRF1 receptor appears to play a critical role in CRF ability to generate panic. Several groups have reported that CRF1 receptor antagonists have anxiolytic properties in rodents (Gehlert et al., 2005; Keck et al., 2001; Keller et al., 2002; Steckler et al., 2006), and panicolytic properties in a rat model of panic disorder (Shekhar et al., 2011). In regard to ORX, CRF-immunoreactive terminals have been shown to contact ORX neurons (Winsky-Sommerer et al., 2004). Although ORX neuron express CRF1 and 2 receptors, local application of the CRF1 receptor antagonist astressin blocks CRF ability to depolarize and increase the firing rate of a subpopulation of ORX neurons (Winsky-Sommerer et al., 2004). Moreover, CRF1 receptor knockout mice have blunted ORX neuronal responses to acute stress (Winsky-Sommerer et al., 2004). In addition to being expressed in PVN neurons that regulated the HPA axis, CRF is also expressed in limbic areas such as the BNST, DRN, and barringtons nucleus (Keegan et al., 1994; Swanson and Simmons, 1989). Of the three areas, the BNST contains the most robust population of neurons that project to ORX neurons (Nambu et al., 1999; Peyron et al., 1998) and may represent the source of CRF to ORX neurons.

Another potent panicogenic stimulus (discussed in detail in section "Orexin role in adaptive response to pH/PCO_2 and in chronic obstructive pulmonary disorder") is increase in arterial carbon dioxide (CO_2), which is a suffocation stimulus (not low oxygen) that provokes strong electrophysiological responses in ORX neurons from local application of high CO_2/low pH solutions (a criteria of a CO_2 chemosensory site). Previous evidence has shown that neurons in the PeF region respond directly to subtle increases in local CO_2/H^+ concentrations (Dillon and Waldrop, 1992). Another recent study determined ORX neurons are highly sensitive to changes in local concentration of CO_2/H^+ which most likely occur through CO_2/H^+-induced closure of leak-like K^+ channels on ORX neurons (Williams et al., 2007). In regard with anxiety, acute higher doses of hypercapnic/normoxic gas exposure (which briefly simulates suffocation) produce marked increases in anxiety-associated behavior and cardiorespiratory responses and also provoke strong ORX neuronal responses in *ex vivo* c-Fos studies (Johnson et al., 2012a). The relevance of stimuli is discussed in detail in section "Orexin role in panic disorder" (as a trigger of panic attacks at low, normally non-panic provoking doses) and in section "Orexin role in adaptive response to pH/PCO_2 and in chronic obstructive pulmonary disorder" as it relates to anxiety and panic associated with chronic obstructive pulmonary disorder (COPD).

Other neurochemical systems that regulate ORX neuronal activity include adenosine receptors which inhibit ORX neurons via adenosine 1 receptors (Liu and Gao, 2007), whereas systemic administration of the adenosine 1 receptor antagonist caffeine (which increases arousal and anxiety) increases neuronal responses in ORX neurons in *ex vivo* c-Fos analyses (Johnson et al., 2012b). Thyrotropin-releasing hormone (TRH) also depolarizes ORX neurons *in vitro*, and injections of TRH into ORX system region increased locomotor activity in control wild-type mice, but not in mice with ORX/ataxin-3 mice where the ORX neurons degenerate postnatally (Hara et al., 2009). Finally, neuropeptide Y, which when administered centrally has anxiolytic effects (Ehlers et al., 1997; Sajdyk et al., 2002, 2008b), inhibits ORX neurons *in vitro* (Fu et al., 2004).

Orexin/hypocretin neurons are predominantly localized to the perifornical hypothalamus, a key panic site

As stated previously, the most striking discovery of these studies was that within the rat brain, the distribution of the ORX-synthesizing neurons was restricted to specific subnuclei in posterior

regions of the hypothalamus. The distribution of ORX-synthesizing neurons was, respectively, described as being restricted to the dorsal and lateral hypothalamic areas (de Lecea et al., 1998), and around the lateral and posterior hypothalamus (Sakurai et al., 1998). However, as illustrated in the inset of Fig. 2a, the distribution of ORX neurons is largely restricted to the (PeF: dorsal to the fornix) with remaining ORX neurons predominantly located in the lateral hypothalamus (LH) and the DMH which collectively contains the dorsomedial hypothalamic nucleus and dorsal hypothalamic area (see distribution of ORX-positive neurons (Johnson et al., 2010b; Nambu et al., 1999; Peyron et al., 1998) combined with delineated nuclei in a recent standard stereotaxic brain atlas (Paxinos and Watson, 2005; also see inset in Fig. 2). This ORX system is highly conserved among vertebrate animals as is the predominant PeF localization of the ORX neurons (humans (Thannickal et al., 2007), primates (Downs et al., 2007), reptiles (Dominguez et al., 2010), amphibians (Lopez et al., 2009a), and fish (Lopez et al., 2009b)).

The PeF role in adaptive panic/"fight-or-flight" responses

There has been a long history of research which has led to the hypothesis that the PeF region is critical in mediating negative emotional responses and cardiovascular components of a panic (or "fight-or-flight") response (Bard, 1928; Bard and Mountcastle, 1948; Hess and Brugger, 1943; Ranson, 1934). In the 1920s, Cannon and Britton found that decorticating cats produced a variety of sympathetic nervous system responses (e.g., increases in blood pressure and stress-associated plasma measures such as NE, epinephrine, and glucocorticoids) and defensive behavior (e.g., hissing, arching of back, and attempts to bite) in response to nonthreatening stimuli, which they referred to as "sham rage" since these responses occurred with little or no provocation. Bard and colleagues later discovered that transections of the forebrain also produced "sham rage," but if the transection were caudal to posterior regions of the hypothalamus that contains the PeF then the "sham rage" responses disappeared (Bard, 1928; Bard and Mountcastle, 1948). This led Bard to propose that the forebrain suppresses emotional responses to inconsequential or trivial stimuli by actions in the posterior regions of the hypothalamus, but when the organism is exposed to an imminent threat (e.g., a conspecific male or predator) then tonic inhibition of this panic-generating region is removed and this produces a "fight-or-flight" response, which could also be labeled "adaptive panic" in contrast to pathological activation of this response. This hypothesis was supported by work done by Walter Hess and colleagues in the 1940s where they electrically stimulated the hypothalamus of awake and freely moving cats and discovered that stimulating some component of posterior hypothalamic regions evoked strong autonomic and somatic responses resembling adaptive panic/defense reactions (e.g., increased blood pressure, piloerection, arching of back) (Hess and Brugger, 1943).

Rodent studies later showed that stimulating the PeF and DMH hypothalamic regions with microelectrodes elicited components of the "fight-or-flight" response such as increases in blood pressure, tachycardia, and hyperventilation (Duan et al., 1994; Markgraf et al., 1991) and flight-associated locomotor behavior that increased with intensity of the stimulation (Duan et al., 1996). More selective pharmacological studies using discrete hypothalamic microinjections (that do not stimulate fibers of passage) showed that stimulating or disinhibiting the PeF/DMH (with excitatory amino acids or the $GABA_A$ receptor antagonist BMI, respectively) initiates similar panic-associated "fight-or-flight" responses (e.g., pressor responses, tachycardia, and increases in locomotion; Anderson and DiMicco, 1990; Samuels et al., 2002; Shekhar and DiMicco, 1987; Shekhar et al., 1990; Soltis and DiMicco, 1992).

These panic-like responses to site-specific stimulation of adjacent structures such as the LH (Shekhar and DiMicco, 1987) or regions dorsal to the PeF/DMH do not result in any cardiovascular response (see review DiMicco et al., 2002). This pattern of autonomic and respiratory responses is similar to responses observed during panic attacks in humans (Liebowitz et al., 1986b), and deep brain stimulation of the posterior hypothalamus (that contains the PeF) of humans also leads to tachycardia and self-reported "panic" (Rasche et al., 2006; Wilent et al., 2010). Collectively, these findings led to this region of the hypothalamus being referred to as the hypothalamic "defense area."

Orexin neurons are highly responsive to stress-related stimuli

Functional *ex vivo* imaging using c-Fos as a nuclear marker for acute increases in neuronal responses following challenges has noted that the ORX neurons in the LH responds to reward-associated cues, whereas the ORX neurons in the PeF/DMH respond to aversive stress-related cues. For instance, increases in c-Fos occur in the LH, but not in DMH/PeF, in response to food, morphine, or cocaine-related cues, yet Harris and colleagues noted that the stress-related stimuli footshock increased c-Fos in the PeF/DMH, but not in LH (Harris et al., 2005; see also review Harris and Aston-Jones, 2006). This is consistent with findings in our lab where panicogenic stimuli (i.e., FG-7142, an inverse benzodiazepine agonist (Johnson et al., 2012b); caffeine, a nonselective competitive adenosine receptor antagonist (Johnson et al., 2012b); and acute hypercapnic gas exposure (Johnson et al., 2012a)) all increase c-Fos responses in ORX neurons in the PeF/DMH, but not in the LH. This activation of the ORX neurons in the PeF/DMH, but not in LH, is also a pattern that has also been observed in an animal model of panic (Johnson et al., 2010b). Overall, these data suggest that there may be some functional differentiation between ORX neurons predominantly located in the DMH/PeF versus ORX neurons in the LH.

Orexin role in mobilizing an integrative anxiety–panic response

ORX role in increasing anxiety states and coordinating an integrative panic/defense response in the presence of an imminent threat or following local disinhibition of the neurons in the PeF region that contains the ORX neurons, is a concept that has emerged slowly in comparison to studies indicating that the ORX system plays a role in sleep–wake cycle, feeding, and reward regulation. In the first 5 years following ORX discovery, initial physiology studies began demonstrating that ORX was released at key sympathetic control centers such as the RVLM and RPa, could increase sympathetic outflow and cardioexcitatory responses (see section "Orexin regulation of panic-like cardiorespiratory activity in respiratory control centers"). Other studies also showed that ORX release in specific parasympathetic control centers such as the nTS and DMX could alter parasympathetic activity (see section "Orexin regulation of the HPA axis"). However, ORX specific role in emotional responses associated with anxiety and panic predominantly occurred later and is discussed in sections "Orexin and anxiety-associated behavior and panic-associated cardiorespiratory responses" and "Anxiogenic/panicogenic effects of ORX in limbic regions (e.g., BNST, CeA, and PVT)."

Orexin and anxiety-associated behavior and panic-associated cardiorespiratory responses

One of the earliest studies to indicate that ORX was a critical substrate in adaptive panic responses was conducted by Kayaba and colleagues, where they disinhibited the PeF (with the $GABA_A$ receptor antagonist, BMI) of wild-type and ORX knockout mice and showed that ORX knockout mice had blunted cardiovascular and

respiratory responses (Kayaba et al., 2003). They also noted that, in comparison with wild-type mice, cardiovascular responses were blunted in ORX knockout mice when they were exposed to a male intruder (in a resident–intruder test). However, a noxious pain stimulus (tail pinch) elicited similar cardiovascular responses in wild-type and ORX knockout mice. This suggests that ORX regulates cardiovascular and respiratory response in specific threatening instances associated with a panic/defense response.

Until recently, there have been few studies that have investigated ORX role in anxiety-associated behaviors using accepted tests for anxiety. However, in 2005, Suzuki and colleagues artificially increased ORX levels in the cerebrospinal fluid (CSF) of mice by injecting ORX-A into the ventricle, which resulted in an increase in anxiety-associated behaviors (i.e., decreased time spent on open, lit areas) in accepted test of anxiety (i.e., light–dark box, elevated plus maze (EPM)), without altering general locomotor activity (Suzuki et al., 2005). Consistent with this, in a clinical study, ORX levels in the CSF were elevated in patients with increased anxiety and panic-associated symptoms, compared to nonanxious patients (Johnson et al., 2010b). The neural systems through which ORX exerts anxiogenic effects are still poorly understood and only now being investigated.

As shown in Fig. 1a (see also Fig. 3c), key limbic brain regions, implicated in anxiety (e.g., BNST and amygdala), receive robust to moderate projections from ORX neurons (Peyron et al., 1998). There is also fairly high expression of ORX1 versus ORX2 receptors in those regions (Marcus et al., 2001). ORX-A application onto BNST and CeA neurons also elicits strong depolarizations *in vitro* (see Fig. 3a and b, unpublished data from A. Molosh). Bisetti and colleagues have shown that ORX-A and ORX-B both equally depolarize many CeA neurons *in vitro* which suggests that the postsynaptic effects could involve ORX1 and/or ORX2 receptors (Bisetti et al., 2006), even though ORX1 receptor expression is much higher than ORX2 receptor in this region (Marcus et al., 2001). In light of ORX strong presence in these anxiogenic brain nuclei, we systemically pretreated rats with either a vehicle or a centrally active ORX1 receptor antagonist (SB334867, 30 mg/kg; Ishii et al., 2005) and then induced anxiety behavior (measured with an open field test and social interaction (SI) test) and panic-associated cardiovascular responses by systemically injecting rats with FG-7142 (an inverse benzodiazepine agonist) (Johnson et al., 2012b). The ORX1 receptor antagonist pretreatment attenuated both anxiety behavior and panic-associated cardiovascular responses (i.e., tachycardia) following FG-7142, thus further support for ORX role in mobilizing anxiety and panic responses. In addition, we used c-Fos immunohistochemistry to map neuroanatomical responses to the FG-7142 ± ORX1 receptor antagonist in critical brain regions implicated in anxiety and panic. We determined that the FG-7142 induced robust increases in cellular responses in the BNST, CeA, PAG subdivisions, and in the RVLM (Johnson et al., 2012b). More importantly, these responses were blocked in rats pretreated with the ORX1 receptor antagonist. This panicolytic effect of ORX1 receptor antagonists is consistent with data shown in sections "Orexin role in panic disorder" and "Orexin role in adaptive response to pH/PCO_2 and in chronic obstructive pulmonary disorder," but this is the first study to demonstrate that the neural circuits through which systemic administration of an ORX1 receptor antagonist could be attenuating panic responses. Although an ORX2 receptor antagonist was not specifically tested for panicolytic effects, there are anatomical and functional data (discussed throughout), suggesting that an ORX2 receptor may also block some components of a full panic response (e.g., the effects of the ORX1 receptor antagonist did not block all aspects of the panic response to FG-7142). In the subsequent paragraphs in this section, we discuss evidence showing that the BNST, CeA, and paraventricular thalamus (PVT, a site that projects heavily to the BNST

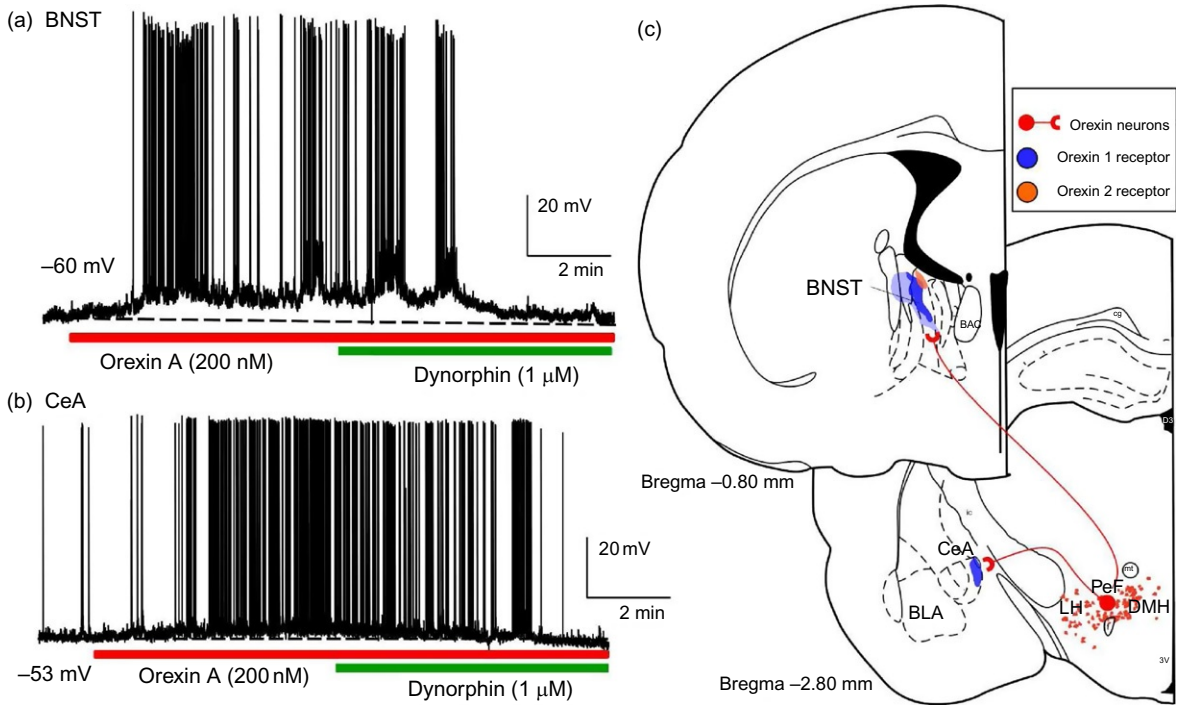

Fig. 3. Representative recordings in current-clamp mode from neurons in the (a) bed nucleus of the stria terminalis (BNST) and (b) central amygdala (CeA) during application of orexin A which induced membrane depolarization and dynorphin which caused a membrane hyperpolarization. (c) Illustrates coronal brain sections showing orexin neurons (red dots in dorsomedial, perifornical, and lateral hypothalamus (DMH PeF and LH)), orexin neuronal projections (red lines) to the BNST and CeA, and the expression of ORX1 (blue shaded areas) and ORX2 (orange shaded areas) receptors in the BNST and CeA. BLA, basolateral amygdala.

and CeA) are important efferent anxiogenic targets for the ORX system. Sections "ORX regulation of panic-like cardiorespiratory activity in respiratory control centers" and "Orexin regulation of the HPA axis" will discuss efferent panicogenic target sites for the ORX system (e.g., RVLM, RPa, nTS, and DMX).

Anxiogenic/panicogenic effects of ORX in limbic regions (e.g., BNST, CeA, and PVT)

As stated previously, there are few studies to date that have thoroughly investigated the neural systems through which ORX exerts anxiogenic effects (that may also contribute to panic-associated cardiorespiratory activity). Recent studies have begun investigating the effects of ORX release in the BNST and CeA on anxiety and panic states. The BNST is critical for anxiety-related (unconditioned) stress responses (Davis and Shi, 1999; Walker and Davis, 1997; Walker et al., 2003). For instance, Hammack et al. found that the typical freezing and increase in escape latency associated with uncontrollable or inescapable shock were also blocked following BNST lesioning (Hammack et al., 2004), while Sullivan et al. found that lesions of this nucleus did not disrupt specific cue-related fear responses, but a more general contextual cue-mediated anxiety (Sullivan

et al., 2004). The CeA and interconnected amygdala nuclei also regulate unconditioned anxiety (Killcross et al., 1997), but in contrast to the BNST, play a clear role in conditioned fear-associated memories (Amano et al., 2010; Kim and Whalen, 2009; Miller and Urcelay, 2007; Tasan et al., 2010; Tye et al., 2011).

Recently, we determined that injecting ORX-A into the BNST increased anxiety-associated behaviors, and this effect appears to be dependent on glutamate (Truitt et al., 2009), which is colocalized in ORX terminals (Henny et al., 2010). There is some evidence that within the BNST the anxiogenic effects are mediated by the ORX1 receptor. In an animal model of panic involving chronic subthreshold disinhibition of the PeF ORX region, we have been able to block interoceptive stressor-induced anxiety behavior by preinjecting an ORX1 receptor antagonist into the BNST (see section "Orexin role in panic disorder" for more evidence of ORX involvement in this model). There is also evidence that ORX may also mobilize panic-associated cardiorespiratory responses through direct effects in the BNST and amygdala. Compared to wild-type mice, ORX/ataxin-3 mice with postnatal loss of ORX neurons have severely blunted cardiorespiratory responses following disinhibition of either the anterior BNST or the amygdala (Zhang et al., 2009). The role of the BNST in panic-associated cardiovascular responses is not entirely consistent and may depend on the rostrocaudal BNST manipulation and/or stress-associated situations. We have previously shown that chronic subthreshold (below dose to acutely provoke anxiety) infusions of a GABA synthesis inhibitor (Sajdyk et al., 2007) or injections of the CRF receptor agonist urocortin 1 (Lee et al., 2008) into the BNST leads to anxiety but not panic vulnerability to an interoceptive stressor (intravenous sodium lactate). This is in contrast to the same treatments where GABA synthesis inhibition in the ORX PeF region (Johnson and Shekhar, 2006; Shekhar and Keim, 1997; Shekhar et al., 2006), or CRF agonist injections into the basolateral amygdala (BLA) (Rainnie et al., 2004) produce anxious rats that display panic-associated cardiovascular responses to mild interoceptive stress. Further, inhibiting the BNST with a $GABA_A$ receptor agonist selectively blocks anxiety behaviors, but not cardiovascular responses provoked with an interoceptive stressor in an animal model of panic vulnerability (Johnson et al., 2008).

There is also evidence that injection of ORX-A, more so that ORX-B, into the CeA region increases anxiety-like behavior (evidenced by decreased time in open areas of the EPM and light–dark box) in hamsters (Avolio et al., 2011). Avolio and colleagues also demonstrated that flunitrazepam, a known anxiolytic, attenuates this response. Although less potent than ORX-A, the anxiogenic effects of injecting ORX-B into the CeA does suggest ORX2 receptor involvement, which is consistent with the electrophysiology data of Bisetti et al. (2006). Orexin also mobilizes anxiety and arousal by indirectly regulating brain regions known to heavily innervate both the BNST and CeA, such as the PVT (Hsu and Price, 2009; Vertes and Hoover, 2008), which may also be an important relay site for anxiety and arousal mobilization by ORX neurons (see review Boutrel et al., 2010). As shown in Fig. 1a, the PVT contains a high density of ORX fibers and also a high expression of ORX1 and 2 receptors. Both ORX-A and -B (ORX-B > ORX-A) also excites PVT neurons that project to the cortex, which may be important for arousal (Huang et al., 2006). Collectively, this led Li and colleagues to hypothesize that ORX release in the PVT increases negative emotional behaviors. In that study, they determined that injections of ORX-A or ORX-B into the PVT increases anxiety- and vigilance-associated behaviors (e.g., decreased exploratory and increased freezing behaviors) in an open field test (Li et al., 2010). Overall, ORX neurons could be mobilizing anxiety behavior and panic responses partially through direct actions onto BNST and CeA neurons, or indirectly through actions on BNST- and CeA-projecting neurons in

the PVT. Given the role that the CeA plays in conditioned fear memory, the strong CeA responses to ORX could indicate the onset of conditioned fear memories (see review Davis and Shi, 1999) and the formation of secondary phobia following initial panic attacks in panic disorder patients (Starcevic et al., 1993a,b). Yet there is little data to date specifically studying ORX role in fear-associated memories.

Orexin regulation of panic-like cardiorespiratory activity in respiratory control centers

Consistent with previous studies supporting ORX cardioexcitatory effects, artificially increasing central levels of ORX leads to marked cardioexcitatory effects. For instance, i.c.v. injections of either ORX-A or ORX-B increase HR and MAP with ORX-A having a greater impact on increases in renal sympathetic nerve activity and plasma NE release (Shirasaka et al., 1999, 2002). Intrathecal (Antunes et al., 2001) or intracisternal (Chen et al., 2000) injections of ORX-A and ORX-B also increases pressor and tachycardia responses. These effects are central, as intravenous injections of ORX-A or ORX-B have no effect on cardiovascular activity (Chen et al., 2000). Here, we will briefly introduce ORX effects on cardiorespiratory responses associated with panic through actions in key autonomic and respiratory nuclei. For a more comprehensive review on the effects of ORX on autonomic and respiratory activity, refer to the review Chapter 4 and also by Kuwaki et al. (2008).

Orexin regulation of sympathetic centers and responses

The RVLM and rostral ventromedial medulla (RVMM) appear to be two critical efferent targets for ORX sympathoexcitatory effects (see Fig. 1a). The RVLM plays a critical role in cardiovascular reflexes associated with MAP and in increasing MAP in response to hypertensive stress (Ross et al., 1984; Yamada et al., 1984). Consistent with a role for the RVLM in PeF/DMH-mediated cardiovascular responses is the finding that pressor responses, elicited from disinhibition of the PeF/DMH, can be severely attenuated by microinjecting the $GABA_A$ receptor agonist muscimol into the RVLM (Fontes et al., 2001). Injecting ORX-A and ORX-B into the RVLM elicits not only pressor responses (Chen et al., 2000; Ciriello et al., 2003; Machado et al., 2002) but also tachycardia in many cases (Chen et al., 2000; Ciriello et al., 2003). ORX depolarizes many RVLM neurons, predominantly through the ORX2 receptor but also through the ORX1 receptor. Huang and colleagues also show that intracisternal ORX2 receptor antagonists are much more effective than ORX1 receptor antagonists on blocking ORX-A-induced depolarizations and intracisternal ORX-A-induced pressor and tachycardia responses (Huang et al., 2010). We have noted significant attenuation of anxiogenic drug (FG7142; Johnson et al., 2012b), anxiogenic stimuli (acute hypercapnia; Johnson et al., 2012a), and interoceptive stress (sodium lactate; Johnson et al., 2010b)-induced cardioexcitation with systemic administration of an ORX1 receptor antagonist (30 mg/kg SB334867), but have not tested ORX2 receptor antagonists. However, the above noted studies in this section suggests that ORX2 receptor antagonists may be even more effective than ORX1 receptor antagonists in blocking cardiovascular responses following stress.

The RVMM, which contains the RPa, is another important relay site for DMH/PeF control of sympathetic outflow and an important efferent target of ORX neurons. Inhibiting the RPa region with muscimol, blocks DMH-evoked tachycardia, which can also be induced by disinhibiting the RPa (Samuels et al., 2002, 2004). Consistent with a role of ORX in this response is that ORX-A (ORX-B not tested) injections into the RVMM selectively increase HR with little effect on MAP (Ciriello et al., 2003).

Orexin regulation of parasympathetic centers and responses

In order for the sympathetic limbs to initiate a simultaneous increase in pressor and tachycardia, the parasympathetically mediated baroreflex must be desensitized (see review McDowall et al., 2006). The dorsomedial medulla contains the nTS and DMX and is a parasympathetic region critical for the baroreflex (Catelli and Sved, 1988). The PeF/DMH directly innervates the nTS (Fontes et al., 2001) and electrical (Coote et al., 1979) or chemical (McDowall et al., 2006) stimulation of the PeF/DMH region overrides or lowers the sensitivity of the baroreflex presumably by regulating activity in the nTS/DMX region. This circuit represents an adaptive means of inhibiting the baroreflex during "fight-or-flight" responses. The nTS and DMX contain numerous GABAergic neurons (Fong et al., 2005) which could be dampening parasympathetic activity by inhibiting local acetylcholinergic preganglionic neurons in the DMX. This notion is supported by evidence where exciting nTS neurons *in vitro* inhibit DMX neurons (Davis et al., 2003). Additional support comes from work on cats where electrical stimulation of the DMH region suppresses the baroreflex via a local GABAergic mechanism in the nTS/DMX region (Sevoz-Couche et al., 2003). Recent studies have shown that ORX may also modulate the baroreflex through actions in the nTS/DMV complex, or indirectly through actions in the RVMM (Ciriello et al., 2003). ORX excites the majority of nTS neurons directly (Yang and Ferguson, 2003; Yang et al., 2003), but also enhances synaptic excitatory input (potentially coreleased glutamate; Smith et al., 2002). Further, ORX may enhance inhibitory input to the DMX arising from the nTS and/or by ORX-mediated synaptic inhibition in the DMX (Davis et al., 2003, 2004). Thus, ORX release in the nTS and DMX (possibly also involving the RVMM) could collectively desensitize the baroreflex to allow sympathetically mediated tachycardia responses.

Orexin regulation of respiratory centers and responses

Many known respiratory control centers such as the pontine PBN/KF, medullary RTN, and Pre-Botz (see review Guyenet et al., 2010) contain ORX fibers (Peyron et al., 1998) and both ORX1 and ORX2 receptors (Marcus et al., 2001). ORX neuronal input onto respiratory control centers also represents polysynaptic input onto motoneurons in the diaphragm (Badami et al., 2010). Consistent with the effects of ORX on most other efferent targets, ORX-A also excites RTN neurons (Lazarenko et al., 2011). Functional studies provide additional support for ORX role in regulating breathing. ORX knockout mice have blunted respiratory responses following disinhibition of the PeF system (Kayaba *et al.*, 2003), which suggests that ORX facilitates respiratory drive under some circumstances. This is supported by data showing that, in urethane anesthetized mice, i.c.v. injections of ORX-A increased respiratory frequency and tidal volume, that also coincided with an increase in blood pressure and heart rate (Zhang et al., 2005). Site-specific microinjections of ORX-B into the pontine respiratory regions such as the KF evokes significant augmentation of the respiratory frequency without altering cardiovascular activity (Dutschmann et al., 2007) and microinjections of ORX-A into the Pre-Botz region increases diaphragm electromyography activity (Young et al., 2005). See Chapter 4 for a comprehensive review on ORX role in breathing regulation and discussion of contribution of different ORX receptors to breathing.

Orexin regulation of the HPA axis

Central ORX release also mobilizes the HPA axis. For instance, i.c.v. injections of ORX-A increases plasma concentrations of adrenocorticotropic hormone (ACTH) and corticosterone *in vivo* (via a CRH receptor-dependent mechanism) and ORX-A directly excites PVN neurons *in vitro* (Samson

et al., 2002). Similar i.c.v. injections of ORX-B was slightly less as potent as ORX-A in increasing plasma ACTH and corticosterone release, which suggests that this response may be primarily ORX2 receptor-mediated (Jaszberenyi et al., 2000; Kuru *et al.*, 2000). The PVN predominantly expresses ORX2 (Marcus et al., 2001; Trivedi et al., 1998), which when antagonized centrally attenuate, but do not block, ORX-A or stress-induced increases in ACTH release (Chang et al., 2007), suggesting that ORX1 receptors may also play a role.

Translational studies linking a hyperactive ORX system to anxiety and panic states

Orexin role in panic disorder

Panic disorder is a severe anxiety disorder characterized by recurrent panic attacks, which are unexpected bursts of severe anxiety that are accompanied by multiple physical symptoms with at least four characteristic symptoms such as tachycardia, hyperventilation, dyspnea, locomotor agitation, etc. (DSM-IV, 1994), and hence often referred to as "spontaneous." Although initially occurring in "spontaneous" manner, panic attacks in patients with panic disorder can be reliably induced in the laboratory by mild interoceptive stimuli (e.g., intravenous (i.v.) 0.5 M sodium lactate or yohimbine (Cowley et al., 1991; Liebowitz et al., 1986a,b) or 7% CO_2 inhalations (Gorman et al., 1994)), suggesting that central pathways that discern threatening versus nonthreatening stimuli lack the necessary inhibitory tone. Consistent with this, reduced inhibitory GABAergic tone may be a critical factor in increased anxiety states and panic attack vulnerability. Genetic polymorphisms in the GABA synthesizing genes (glutamic acid decarboxylase) are associated with vulnerability to panic disorder (Hettema et al., 2005), and altered benzodiazepine binding (Bremner et al., 2000) has been reported in the brain of subjects with panic disorder. Further, benzodiazepines, which are the most effective panicolytic treatment (Baldwin et al., 2005; Bandelow et al., 2008; Cloos and Ferreira, 2009; Nutt et al., 2002), restore GABAergic inhibition (Goddard et al., 2004). Overall, loss of GABAergic tone in a panic-generating CNS site(s) may be a major contributing factor in panic vulnerability to normally innocuous interoceptive or exteroceptive stimuli. The posterior regions of the hypothalamus are one of the earliest activated brain areas during the onset of a panic attack (Boshuisen et al., 2002).

Critical panic-generating sites have been identified in the CNS of rats, where acute and abrupt inhibition of GABAergic tone leads to anxiety behavior and panic-associated cardiorespiratory and locomotor responses. These include, in addition to the PeF/DMH (see section "Orexin/hypocretin neurons are predominantly localized to the perifornical hypothalamus, a key panic site" for details), the BLA and the dorsal periaqueductal gray (see review Shekhar et al., 2003). There are other potential sites that are as yet not fully explored including LS, medial preoptic area, and possibly sites in frontal cortex (Anantha Shekhar et al., unpublished results). These brain regions are significantly more activated in neuroimaging studies during a panic attack in panic disorder (Boshuisen et al., 2002). These clinical and preclinical observations led to a rat model of panic disorder that was developed by Shekhar and colleagues that involved chronic subthreshold inhibition of GABA tone in the PeF/DMH, which contains the majority of ORX neurons. Specifically, chronic reduction of GABA synthesis in the PeF/DMH of rats using L-allylglycine (L-AG) produces anxiety-like states (measured by SI and EPM anxiety tests) and a vulnerability to panic-like responses (cardiorespiratory stimulation and flight-like locomotion) following i.v. infusions of 0.5 M sodium lactate (Johnson and Shekhar, 2006; Johnson et al., 2008; Shekhar and Keim, 1997, 2000; Shekhar et al., 1996, 2006), thus providing a model of human panic disorder.

Recently, we sought to confirm ORX role in this model of panic disorder. Initial studies

confirmed that chronically removing inhibitory GABAergic tone in the DMH/PeF (to produce panic-prone rats) selectively increased local ORX neuronal activity that was correlated with anxiety states (Johnson et al., 2010b) and suggested that ORX may be a key substrate mediating panic-like responses in this animal model of panic disorder. We then systemically pretreated panic-prone rats with a centrally active (Ishii et al., 2005), ORX1 receptor antagonist (SB334867, 30 mg/kg) and attenuated the anxiety-like behavior (reduced SI), locomotor, and cardioexcitatory responses induced by the lactate challenge (Fig. 4). Similarly, another ORX1 receptor antagonist (SB408124, 30 mg/kg, Tocris) also attenuated the sodium lactate-induced increases in locomotor activity and tachycardia responses in another group of panic-prone rats when compared to vehicle. We noted no significant side effects of the ORX1 receptor antagonist on sedation that was assessed by

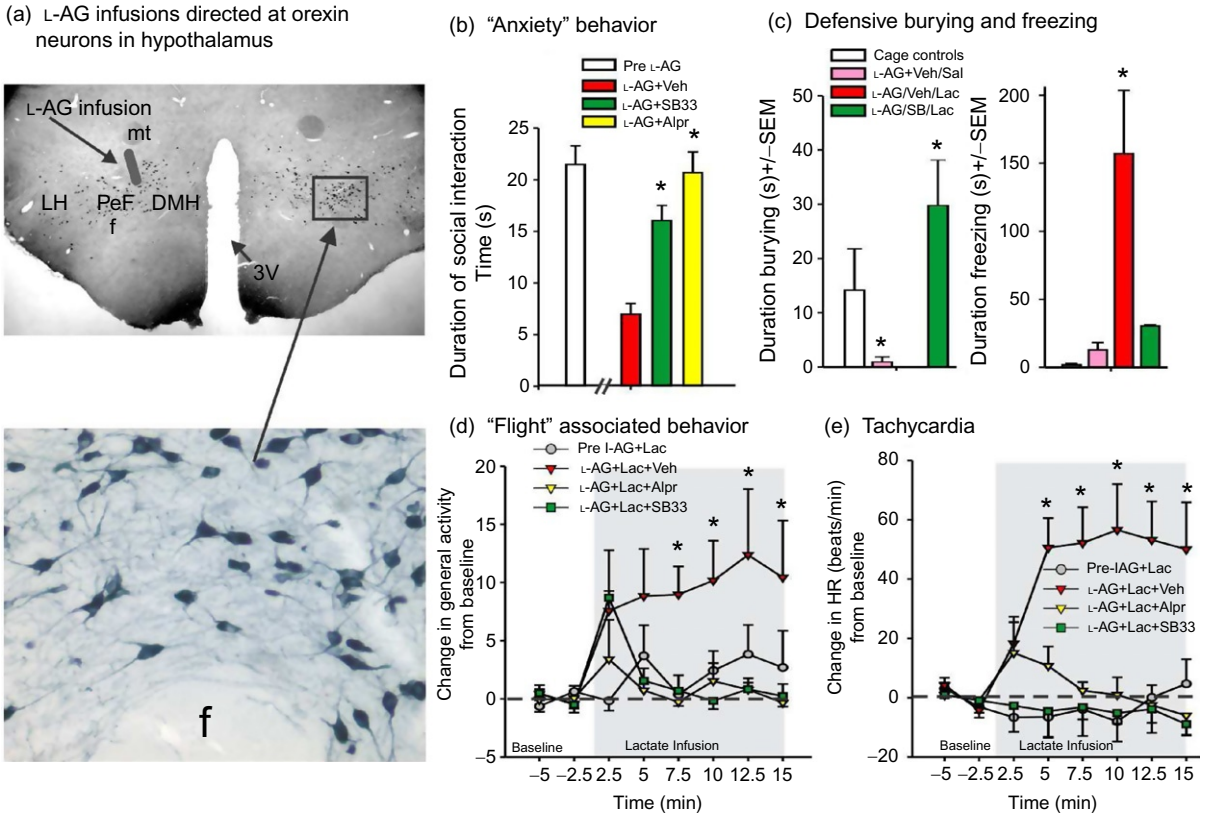

Fig. 4. In an animal model of panic vulnerability [involving GABA synthesis inhibition in the ORX system using L-allylglycine (L-AG), Fig. 1a shows cartoon of injection site on coronal hypothalamic rat brain sections immunostained for ORX-A], prior systemic injections of a centrally active ORX1 receptor antagonist (SB334867, 30 mg/kg Tocris) or benzodiazepine (Alprazolam, 3 mg/kg, Sigma) attenuated sodium lactate provoked (b) "anxiety" behavior in social interaction test, (c) defensive burying and freezing behavior in defensive shock probe test (alprazolam not done here), (d) "flight" associated locomotion, and (e) tachycardia. * indicates significant effects between groups using a Fisher's LSD *post hoc* test protected with an ANOVA, $p < 0.05$. DMH, dorsomedial hypothalamus; f, fornix; LH, lateral hypothalamus; mt, mammillothalamic tracts. Figure 4c adapted by permission from Johnson et al. (2010b).

monitoring baseline locomotion or autonomic activity. These effects of ORX antagonists were similar to alprazolam, a clinically effective antipanic drug that blocks spontaneous and i.v. lactate-induced panic attacks (Cowley et al., 1991; Liebowitz et al., 1986a). Further, anxiolytic effects of benzodiazepines could be partially due to direct effects on ORX neurons, based on c-Fos studies where anxiolytic doses of diazepam inhibits ORX neuronal activity (in both the PeF/DMH and the LH; Panhelainen and Korpi, 2011), whereas panicogenic doses of the inverse benzodiazepine agonist increases activity in ORX neurons (in PeF/DMH, but not in LH; Johnson et al., 2012b). We also confirmed ORX role in panic responses to sodium lactate, but locally silencing the ORX precursor gene in the PeF region.

Interestingly, chronic treatment with sertraline, a well-known antipanic and antidepressant drug, was reported to reduce mean ORX levels in the CSF, whereas bupropion, an antidepressant with a lower efficacy in treating panic disorder failed to reduce CSF ORX levels in human subjects, suggesting that ORX reduction as a possible mechanism for antipanic effects of certain antidepressants (Salomon et al., 2003). Thus, aberrant functioning of the ORX system in the DMH/PeF region may underlie vulnerability to panic-like responses and that ORX1 receptor antagonists may provide a novel therapeutic approach for the treatment of such severe anxiety disorders.

Orexin role in adaptive response to pH/PCO_2 and in chronic obstructive pulmonary disorder

Orexin neurons and adaptive responses to hypercapnia

As mentioned in section "Anxiety and panic-related neurochemical input onto ORX neurons" and illustrated in the inset of Fig. 1b, ORX neurons are highly sensitive to local changes in CO_2/pH. Normally, blood CO_2/H^+ is maintained within a very narrow range, and mild arterial elevations of CO_2 (i.e., hypercapnia), that can occur from hypoventilation or in some respiratory disorders such as COPD, initially leads to an increase in respiratory activity to help "blow off" excess CO_2 (see review Guyenet et al., 2010). Carbon dioxide crosses the blood–brain barrier easily (Forster and Smith, 2010; Fukuda et al., 1989) to directly interact with specialized CO_2/H^+ chemosensory neurons in the medulla that are critical for regulating breathing following subtle changes in CO_2/H^+ (Guyenet et al., 2010). However, if CO_2 levels continue to increase, this leads to sense of "suffocation" that is accompanied by adaptive behavioral and autonomic responses which help restore homeostasis. For instance, exposing rats to mild hypercarbic gas (e.g., 7% CO_2; Akilesh et al., 1997) increases respiratory activity that reduces hypercapnia without mobilizing other components of panic. However, exposing rats to higher concentrations of hypercarbic gas (e.g., $\geq$10% CO_2) elicits additional components of panic-associated responses as evidenced by increases in sympathetic activity (Elam et al., 1981), blood pressure (Walker, 1987), and anxiety-like behaviors (Cuccheddu et al., 1995; Johnson et al., 2010a). In humans, a single breath of air containing 35% CO_2 increases anxiety and sympathetic–adrenal responses (Argyropoulos et al., 2002; Griez and Van den Hout, 1983; Kaye et al., 2004) and inhaling 7.5% CO_2 for 20 min leads to increases in anxiety and cardiorespiratory responses (Bailey et al., 2005). Therefore, severe hypercapnia-induced anxiety responses and autonomic hyperactivity could be relevant to managing hypercapnic conditions such as COPD, asthma, or bronchitis.

Similar to chemosensory medullary neurons, ORX neurons also display CO_2/H^+-sensitive properties, but with lesser chemosensitivity (Williams et al., 2007), suggesting that they may respond to only panic threshold hypercapnia where they may play a role in hypercapnia-induced anxiety and cardiorespiratory responses. In support of this hypothesis, ORX knockout mice also have blunted respiratory responses to 5–10% hypercarbic,

normoxic gas exposure, and injecting wild-type mice with an ORX1 receptor antagonist attenuates hypercapnia-induced respiratory responses (Deng et al., 2007). Dias and colleagues later showed that injecting an ORX1 receptor antagonist into the RTN of rats also blunts respiratory responses to 7% CO_2 with balanced air but noted that this effect was more prominent in awake (~20% reduction) versus sleeping (~9% reduction) rats (Dias et al., 2009). In a recent series of studies, we wanted to assess ORX role-mobilizing anxiety behavior and panic-associated cardiovascular responses to doses of hypercapnia (>10%) known to provoke panic. We determined that systemically pretreating rats with an ORX1 receptor antagonist (SB334867, 30 mg/kg) attenuated hypercapnic (20%), normoxic gas exposure (5 min of ramping ambient CO_2 concentrations to 20% at the 5-min time point, followed by rapid clearance)-induced anxiety behavior, and blocked pressor responses, without altering a robust bradycardia (Johnson et al., 2012a). This suggests that CO_2-mediated bradycardia does not involve an ORX1 receptor-dependent mechanism (we did not rule out ORX2 receptor involvement). Locomotor activity was unaffected by the hypercarbic gas exposure, which suggests that this challenge was not anesthetizing or sedating the rats. Another surprising result was that compared to vehicle-treated rats, the ORX1 receptor antagonist also did not alter respiration rate increases during the hypercapnia challenges but did reduce respiration rate following the offset. In conscious rats, 20% hypercapnia exposure caused an increase in the respiratory rate from ~120 to ~150 bpm that became more paced during the hypercapnia exposure when the rat had less locomotor activity. Then at the offset of the hypercapnic gas, the respiratory rate increased from ~150 to >200 bpm, which coincided with an increase in sniffing and locomotor behavior. This suggests that the ORX1 receptor antagonist is not directly altering respiratory drive, but rather the behavioral arousal posthypercapnia exposure. Although this study was conducted in conscious rats, the study was done during the inactive period when CSF levels of ORX are lowest during the 24-h period (Desarnaud et al., 2004), and where other studies have seen little effect of ORX on respiratory responses to hypercapnia. For instance, ORX regulation of respiration in response to hypercapnia appears to be dependent on whether the studies are done during the wake or sleep periods of the animal. Kuwaki and colleagues demonstrated that ORX knockout mice had blunted respiratory responses to 5% and 10% CO_2 exposure during wakefulness but not during sleep states (Kuwaki et al., 2008). Nattie and Li saw similar state-dependent effects of ORX, where systemic injections of the dual ORX antagonist almorexant decreased respiration responses to exposure to 7% CO_2 but only during wakefulness (Nattie and Li, 2010). Both these studies used 10% or lower concentrations of CO_2, which could below the panic-inducing 20% concentration used in our study. Thus, ORX appears to be involved in the regulation of hypercapnia-induced respiratory responses most potently during conscious wake periods and during periods of heightened behavioral activity or danger.

Orexin role in respiratory disorders such as COPD

Subjects with episodes of hypercapnia (such as patients with COPD, bronchitis, or asthma) have significant comorbidity with severe anxiety and sympathetic arousal, which can make management of these symptoms difficult, because potent anxiolytics such as benzodiazepines also suppress respiratory drive which is needed to blow off CO_2 during hypercapnic episodes. Our results suggest that the ORX system may play an important role in these responses to hypercapnia, particularly concomitant severe anxiety. Preclinical modeling of COPD and clinical COPD has also recently been linked to a hyperactive ORX system. In a preclinical study, COPD was modeled in rats by exposing them to chronic cigarette smoke (1 h, twice/day over 12 weeks) (Liu et al., 2010). By week 12, the COPD rats, compared to control rats, had (1)

COPD-associated lung pathology (i.e., coalesced alveoli and thickened bronchiolar walls); (2) >100% increase in hypothalamic and medullary ORX-A protein expression; and (3) heightened phrenic nerve responses to ORX-A injections into the Pre-Botz. Further, in a recent clinical study, ORX-A, which crosses blood–brain barrier easily (Kastin and Akerstrom, 1999), was increased threefold in the plasma of patients with COPD and hypercapnic respiratory failure, compared to controls (Zhu et al., 2011). Therefore, ORX1 receptor antagonists may represent a novel anxiolytic treatment for COPD patients that experience anxiety. Further, ORX1 receptor antagonists also reduce hypertensive responses due to hypercapnia, which may also be exacerbated by the use of sympathomimetics and bronchodilators in COPD. Doses of the ORX1 receptor antagonist used here were anxiolytic and panicolytic without inducing somnolence. We have also previously shown that the dose of the ORX1 receptor antagonist used here does not alter baseline MAP, HR, or locomotion in untreated control rats (Johnson et al., 2010b). A caveat is that we did not look at long-term effects of repeated use of the ORX1 receptor antagonist which may alter wakefulness and baseline cardiorespiratory activity. Thus, the ORX system may also be an important target in future management of COPD and other hypercapnic conditions.

ORX role in mobilizing panic responses to more severe hypercapnia may also be relevant to panic disorder patients, where exposing these patients to CO_2 at concentrations below the panic threshold elicits panic attacks in the majority of these patients. For instance, mild hypercapnia (5–7% CO_2), that is normally below the threshold provokes panic and anxiety responses, elicits panic attacks in the majority of patients with panic disorder compared to few healthy controls (Goetz et al., 2001; Gorman et al., 1984, 1988). This led Klein to propose that the "suffocation"/CO_2 monitors in the brain of some patients with panic disorder are hypersensitive to CO_2 and lead to panic responses to slight changes in ambient CO_2 (Klein, 1993). In a recent review, Freire and colleagues discuss supporting evidence for panic vulnerability to CO_2 in subtypes of panic disorder with comorbid respiratory symptoms (Freire et al., 2010). Preclinical and clinical studies will need to further confirm this phenomenon and determine whether the ORX system may play a role.

Orexin role in posttraumatic stress disorder and phobias

As discussed in section "Orexin regulation of panic-like cardiorespiratory activity in respiratory control centers," ORX ability to excite amygdala nuclei (Bisetti et al., 2006; see also Fig. 3) suggests ORX may regulate fear conditioning which plays a role in phobias and posttraumatic stress disorder (PTSD). The amygdala is strongly linked to conditioned fear (Tye et al., 2011), and pathology is associated with PTSD (see review Mahan and Ressler, 2012). Surprisingly, there is little preclinical data investigating ORX role in fear conditioning. Yet, there is additional preclinical data supporting a role for ORX in the fear conditioning. Neurotoxic lesions of the PeF region severely attenuated fear conditioned behavior (i.e., freezing and ultrasonic vocalizations) and panic-associated cardioexcitatory responses (pressor and tachycardia activity) (Furlong and Carrive, 2007). Clinical studies have linked a hyperactive ORX system to increased anxiety states, but the duration of the anxiety and comorbid depression may lead to hyperactive ORX activity.

Recently, Ponz and colleagues demonstrated that amygdala activity during aversive conditioning is reduced in humans with narcolepsy (Ponz et al., 2010), a condition strongly associated with dramatic loss of ORX neurons (Peyron et al., 2000; Thannickal et al., 2000). We have shown that heightened states of anxiety humans are associated with increased CSF levels of ORX-A (Johnson et al., 2010b), which suggests that hyperactive ORX system may lead to increased vulnerability to the development of phobias or PTSD in the presence of trauma. In a recent clinical study on PTSD related

to combat, Strawn and colleagues assessed ORX-A level in the CSF and plasma predicting to see high levels. However, they found that ORX-A levels were reduced in PTSD patients and also correlated with the severity of PTSD symptoms (Strawn et al., 2010). However, Strawn and colleagues did not specifically assess depression symptoms, which are associated with reduced central ORX tone, even in the presence of comorbid anxiety. Specifically, clinical data have shown that depression or comorbid depression and anxiety are associated with low levels of CSF ORX-A (Brundin et al., 2007; Johnson et al., 2010b). Based on these observations, short-term stress and anxiety states may be associated with increased ORX activity, whereas chronic stress could lead to low ORX activity. A preclinical study conducted by Marcus and colleagues appears to support this hypothesis. In that study, they noted that ORX-A levels were increased in the CSF of rats after an acute forced swim stress but were decreased in rats following long-term immobilizations (no changes were noted with cold stress, or acute immobilization; Martins et al., 2004).

In a recent reanalysis of a study in Johnson et al. (2012a), we assessed the induction of contextual fear-associated behaviors in a defensive burying test study that included rats that had chronic disinhibition of ORX pathways which induces panic vulnerability (Johnson et al., 2010a). The panic-prone rats with chronic disinhibition of ORX neurons (L-AG infusion into DMH/PeF) as opposed to the controls (D-AG-infused animals) received significantly lower number shocks (Fig. 5a), yet developed greater

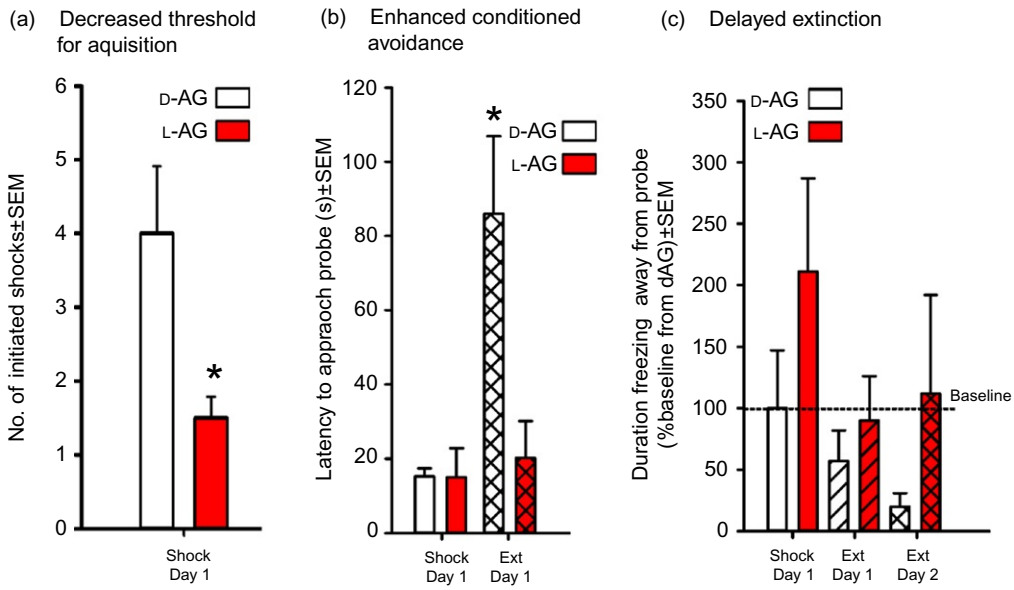

Fig. 5. Compared to control rats with intact GABA in the dorsomedial/ perifornical hypothalamus (DMH/PeF, receiving inactive GABA synthesis inhibitor locally, D-AG), panic-prone rats with disrupted GABA tone in the DMH/PeF (from 5 days of local L-allylglycine L-AG infusions) had (a) decreased thresholds for acquisition of aversion to electrified shock probes; (b) enhanced conditioned avoidance of nonelectrified shock probes on extinction Day 1; and (c) delayed extinction which was evidenced by the duration of freezing away from nonelectrified shock probe over testing days. * indicates $p < 0.05$ using two tailed t-test.

avoidance responses on the following day when tested for conditioned fear (Fig. 5b). During the extinction trial 24 h later, the L-AG animals showed significant delay in normal extinction responses (Fig. 5c). These data clearly suggest that the panic-prone rats, despite greater avoidance of shock, exhibit rapid induction, greater severity, and persistence of conditioned fear to contextual cues. We also have recent preliminary evidence that ORX may be implicated in robust acquisition of conditioned fear in a classical auditory cue induced pavlovian-conditioned fear paradigm (Anantha Shekhar et al., unpublished results). All of these data further support that activation of the ORX system during fearful situations could enhance acquisition of conditioned fear, leading to phobias and PTSD-like consequences.

Concluding remarks

Under nonstressful condition, ORX main role appears to be maintaining wakefulness and increasing vigilance and arousal during routine goal-oriented behavior. However, when confronted with threatening stress-related challenge, ORX also mobilizes an adaptive and integrative stress response that is comprised of anxiety-associated behavior, cardiorespiratory, and endocrine responses. There is also emerging evidence that the dysregulation of the ORX system contributes to pathologies associated with anxiety and depression and potentially pathology associated with fear-associated memory (e.g., PTST and phobias).

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