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Activation of orexin/hypocretin neurons is associated with individual differences in cued fear extinction☆

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HIGHLIGHTS

- Outbred rats show individual differences in cued fear extinction.
- Orexin neurons in the medial hypothalamus are activated during fear extinction.
- Greater activation of orexin neurons in hypothalamus is correlated with freezing during fear extinction.

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ABSTRACT

Identifying the neurobiological mechanisms that underlie differential sensitivity to stress is critical for understanding the development and expression of stress-induced disorders, such as post-traumatic stress disorder (PTSD). Preclinical studies have suggested that rodents display different phenotypes associated with extinction of Pavlovian conditioned fear responses, with some rodent populations being resistant to extinction. An emerging literature also suggests a role for orexins in the consolidation processes associated with fear learning and extinction. To examine the possibility that the orexin system might be involved in individual differences in fear extinction, we used a Pavlovian conditioning paradigm in outbred Long-Evans rats. Rats showed significant variability in the extinction of cue-conditioned freezing and extinction recall, and animals were divided into groups based on their extinction profiles based on a median split of percent freezing behavior during repeated exposure to the conditioned cue. Animals resistant to extinction (high freezers) showed more freezing during repeated cue presentations during the within trial and between trial extinction sessions compared with the group showing significant extinction (low freezers), although there were no differences between these groups in freezing upon return to the conditioned context or during the conditioning session. Following the extinction recall session, activation of orexin neurons was determined using dual label immunohistochemistry for cFos in orexin positive neurons in the hypothalamus. Individual differences in the extinction of cue conditioned fear were associated with differential activation of hypothalamic orexin neurons. Animals showing poor extinction of cue-induced freezing (high freezers) had significantly greater percentage of orexin neurons with Fos in the medial hypothalamus than animals displaying significant extinction and good extinction recall (low freezers). Further, the freezing during extinction learning was positively correlated with the percentage of activated orexin neurons in both the lateral and medial hypothalamic regions. No differences in the overall density of orexin neurons or Fos activation were seen between extinction phenotypes. Although correlative, our results support other studies implicating a role of the orexinergic system in regulating extinction of conditioned responses to threat.

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1. Introduction

The formation of aversive memories following exposure to stressful or harmful stimuli is a natural and necessary process for survival. However, when fear memories take on excessive salience or extinction of learned fear is dysregulated, trauma or stress-related disorders, such as post-traumatic stress disorder (PTSD), can manifest. While somewhere between 50 and 84% of the general population will experience

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a traumatic event, most individuals are resilient to these stressors, and estimates suggest that somewhere around 10% of the population will go on to develop PTSD [1–4]. This suggests that there are individual neurobehavioral differences that contribute to either resiliency from or susceptibility to the long-term negative effects of stress. Animal models of fear extinction have emerged as a tenable way to understand the neurobiological, genetic, and epigenetic mechanisms that drive individual differences in risk and resilience following traumatic stress [5,6]. Moreover, PTSD patients show impaired fear extinction learning and retention responses (see [5–9] for review), and emerging evidence suggests that individual differences in extinction of learned fear may predict susceptibility to stress disorders such as PTSD [10,11].

Preclinical studies have described an analogous variability in extinction learning and recall among rodents [6,12–17], making them a potentially useful model for examining the individual neurobiological differences that may underlie susceptibility to long term consequences of traumatic stress. In fear conditioning procedures animals are conditioned by pairing a neutral stimulus, such as a tone (the conditioned stimulus or CS) with an aversive stimulus such as a footshock (the US or unconditioned stimulus). The pairing of the CS and US enables both the context and the CS (the tone), even when the CS is presented in a novel context, to elicit a defensive response such as freezing [18]. Repeated re-exposure to the context or the CS in the absence of the US results in the extinction of the response [19]. A variety of studies have demonstrated key roles for plasticity in the amygdala, hippocampus and prefrontal cortex in driving these conditioned fear and extinction responses [5,18,19]. Moreover, studies suggest that extinction learning involves distinct neuronal populations and signaling processes from the original learning of the contextual or cue-conditioned responses [20,21], and extinction of contextual or cued fear responses appears to involve prefrontal-amygdalar and prefrontal-hippocampal circuits (see [19,22,23]).

Another system that has emerged as an additional modulator of not only arousal and attention, but fear learning and extinction, is the orexin system [24–29]. The orexin/hypocretin family of neuropeptides were discovered in the late 1990s [30,31] and has a well-established role as a physiological integrator in the control of sleeping and homeostatic regulation, as well as attention, arousal, and stress responses [25,26,32–35]. Two peptides, orexinA/hypocretin1 [OxA] and orexinB/hypocretin2 [OxB] are produced by the preproorexin gene, and act on two G protein-coupled receptors. The orexin/hypocretin 1 receptor [Ox1R/HcrtR1] is selective for OxA, while the orexin 2 receptor [Ox2R/HcrtR2] binds both OxA and OxB with high affinity [31]. Although restricted to the hypothalamus, orexin neurons have extensive projections throughout the central nervous system [36] and orexin receptors are found throughout the brain [26,37–39]. Orexin projections are particularly dense to several brain regions known to be critical in fear learning and extinction, including the locus coeruleus (LC), amygdala, prefrontal cortex (PFC), and paraventricular thalamus (PVT) [36]. Orexin neurons also receive afferent projections from many of these same brain regions, in addition to projections from the brainstem [40,41].

Several lines of evidence implicate orexins and orexin receptors in the threat circuit in mediating defensive responses in unconditioned behavioral tasks, as well as serving a modulatory role in the consolidation of fear memories and fear extinction. Activation of these neurons and orexin effects, however, seems to be more associated with stressors that induce arousal and attentional processes associated with environmental stimuli [42–44]. Administration of orexins or optogenetic stimulation of these neurons produce defensive responses or anxiogenic effects in several tasks [45–51]. Studies examining expression of immediate early genes, such as *cfos*, have demonstrated that orexin neurons are activated by anxiety-related or threat stimuli, or anxiogenic drugs such as FG-7142 or caffeine [25,52,53]. Acute unconditioned stressors [27,33,42,54–57], as well as chronic unpredictable stress [58] and sodium lactate infusions that precipitate a panic-like state [59] induce

activation of orexinergic neurons particularly in the dorsomedial or perifornical [60] hypothalamic regions. Activation of orexin neurons following exposure to a conditioned context has been seen in some, but not all studies [27,42,55], and extinction training with repeated exposures to the conditioned context or cues also activates orexin neurons [24,61]. Pharmacological studies suggest that administration of OxA attenuates fear extinction [24], while orexin receptor antagonists or orexin receptor genetic ablation block the consolidation of fear learning and accelerate fear extinction [24,26,28,29]. These studies have also demonstrated unique roles for Ox1 and Ox2 receptors in different brain areas in these processes [26]. Individual differences in expression of preproorexin mRNA following footshock are also correlated with freezing during re-exposure to the conditioned context, and these changes in preproorexin gene expression are more pronounced in animals that show enhanced response to novel sound cues after footshock stress [27]. Human studies have also demonstrated amygdalar orexin release is associated with emotional arousal [62].

Therefore, the aim of this study was to determine if there are correlations between activation of orexin neurons in the hypothalamus, and individual differences in extinction learning or recall. Using outbred Long-Evans rats, we examined neuronal activation using *cfos* (Fos) protein expression in OxA-positive neurons of the hypothalamus associated with individual differences in extinction of cue-induced conditioned freezing. Our results indicate animals showing poor extinction learning and recall (high freezers) had significantly more activation of orexin neurons in the medial hypothalamus than low freezers, suggesting that greater orexinergic activity is associated with resistance to fear memory extinction. Our results add to a growing literature suggesting the orexin system represents a target for understanding the neurobiological underpinnings of the individual differences in fear extinction, as well as susceptibility to the long term consequences of traumatic stress.

2. Methods

2.1. Subjects

Adult male Long Evans rats (175–200 g; Harlan, Indianapolis, IN) were singly housed and maintained on a 12-hour light dark cycle (lights on at 7 AM) with ad libitum access to food and water. After arrival in the vivarium, animals were habituated to daily handling for at least one week before the experiment. All procedures were approved by the University of South Carolina Institutional Animal Care and Use Committee. Twenty-three animals were tested in two cadres to provide tissue for immunohistochemical processing. Animals were tested in a cylindrical chamber for unconditioned freezing and other behaviors one week prior to fear conditioning (data not shown).

2.2. Fear conditioning and extinction

For examining conditioned fear and extinction, a protocol modified from Likhtik et al. (2008) was used [63]. For acquisition of conditioned fear, male Long-Evans rats ($N = 23$) were placed in a shock box (Context A; Med Associates, Inc) within a sound-attenuating box containing a ventilation fan and a house light. Unconditioned freezing was recorded for 3 min. Rats were then conditioned with three 10 s tones (80 dB, 2 KHz) co-terminating with a 1 mA footshock (1 s) presented at 60 s intervals. The shock box chamber was cleaned with mild (7%) ammonium hydroxide solution between animals. On day two (24 h post-acquisition) rats were returned to the shock box (Context A) for 8 min without tone or shock for assessing context-conditioned freezing. On day 3 (48 h post-acquisition) to assess cue-conditioned freezing and extinction learning, animals were placed in a completely different context in a sound-attenuated box in a separate testing room (Context B) that consisted of a clear Plexiglas bowl with distinct visual and olfactory cues (20 μ L lemon extract) compared to the conditioning Context A,

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