Physiology & Behavior 130 (2014) 182–190

Contents lists available at ScienceDirect

Physiology & Behavior

journal homepage: www.elsevier.com/locate/phb

Optogenetic examination identifies a context-specific role for orexins/hypocretins in anxiety-related behavior

W. Heydendael [a,b,*], A. Sengupta [a], S. Beck [a,b], S. Bhatnagar [a,b]

[a] Children's hospital of Philadelphia, United States
[b] University of Pennsylvania, United States

HIGHLIGHTS

- Activation of orexin system increases anxiety-related behavior in novel context
- Activation of orexin system increases exploration and arousal only in novel environment
- Orexin-mediated increases in anxiety and arousal associated with orexin activity in PVT and LC

ARTICLE INFO

Article history:
Received 18 January 2013
Received in revised form 23 August 2013
Accepted 10 October 2013
Available online 18 October 2013

Keywords:
Optogenetics
Orexins
Hypocretins
Social interaction
Anxiety
Stress

ABSTRACT

Maladaptation to stress is associated with psychopathology. However, our understanding of the underlying neural circuitry involved in adaptations to stress is limited. Previous work from our lab indicated the paraventricular hypothalamic neuropeptides orexins/hypocretins regulate behavioral and neuroendocrine responses to stress. To further elucidate the role of orexins in adaptation to stress, we employed optogenetic techniques to specifically examine the effects of orexin cell activation on behavior in the social interaction test and in the home cage as well as orexin receptor 1 internalization and ERK phosphorylation in brain regions receiving orexin inputs. In the social interaction test, optogenetic stimulation of orexin neurons decreased time spent in the interaction zone while increasing the frequency of entries into the interaction zone. In addition, optogenetic stimulation of orexin neurons increased the total distance traveled in the social interaction arena but had no effect on their home cage behavior. Together, these results suggest that orexin release increases anxiety in the social interaction test while increasing the salience of novel but not familiar environmental stimuli. Consistent with activation of orexin neurons, optogenetic stimulation increased orexin receptor1 internalization and ERK phosphorylation in the paraventricular thalamus (PVT) and locus coeruleus (LC), two regions heavily innervated by orexin neurons. Together these results show for the first time that elevation of orexin activity, possibly in the PVT and LC, is associated with increased anxiety, activity, and arousal in a context-specific manner.

1. Introduction

Orexins (also called hypocretins) are neuropeptides exclusively produced in cells of the lateral and posterior hypothalamus [7,28]. Orexins are synthesized from the precursor molecule pre-pro-orexin that is cleaved into two structurally related and highly conserved peptides, orexinA and orexinB [7,28] that bind to two G-protein coupled receptors, orexin1 (orexin1R) and orexin2 receptors (orexin2R). Stimulation of orexin receptors promotes arousal or a heightened responsiveness to sensory inputs and increased wakefulness [29]. An obvious extension of the role of orexins in initiating arousal is increasing the salience of novel potentially threatening environmental cues. Previous work from our lab has indicated a role for orexins acting in the posterior paraventricular thalamus (PVT) in regulating the stress response and increasing anxiety-related behavior in repeatedly stressed rats [13,19]. Other work has reported that acute intraventricular (ICV) or systemic administration of orexins can also increase anxiety-related behavior in the open field test [14,33], depolarize cells [13], and increase CRH and AVP mRNA in the paraventricular hypothalamus (PVN), the hypothalamic arm of the hypothalamic pituitary adrenal (HPA) axis [6,31]. These increases in CRH and AVP are also consistent with orexin-induced increases in plasma ACTH and corticosterone [4,17,31]. However, it is unclear whether endogenous orexins induce anxiety in all contexts. Further, little is known about the possible neural substrates for these actions. In order to further elucidate the role of orexins in anxiety related behavior we employed optogenetic techniques to specifically activate orexin cells. This technique allows for specific temporal resolution in activating the orexin system and produces the release of orexins within the physiologic capabilities of the cell [1].

* Corresponding author at: 1029 Federal st.Philadelphia, PA 19147, USA.
E-mail address: Heydenw@gmail.com (W. Heydendael).

0031-9384/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.physbeh.2013.10.005

Effects of orexin cell activation on behavior in the social interaction test and home cage were examined. To determine the brain regions that could be the site of orexin actions, orexin receptor1 internalization, ERK phosphorylation, and cFos expression were quantified in three specific brain regions receiving orexin inputs following optogenetic stimulation of orexin neurons. These three brain regions were the PVT, the locus coeruleus (LC), and prefrontal cortex (PFC). When taken together, the results presented here suggest that orexins mediate context-specific actions on anxiety and arousal and that the paraventricular thalamus (PVT) and locus coeruleus (LC) are two brain regions important in mediating these effects.

2. Methods

2.1. Animals

Male Sprague–Dawley rats (Charles River, Kingston, NY) weighing between 225 and 250 g were given ad libitum access to food and water and individually housed in plastic tub cages on a 12:12 h light: dark cycle with lights on at 0600 h. A 5–7 day acclimation period was allowed prior to start of experimentation and surgery. All procedures were approved by the IACUC at the CHOP Research Institute.

2.2. Surgery

Rats were anesthetized using a cocktail containing ketamine, xylazine, and acepromizine. Using stereotaxic techniques, unilateral guide cannulae (26ga) were implanted in the lateral posterior hypothalamus, the site of orexin cell bodies, with the following coordinates (from bregma): AP: −2.8, ML: −1.8 mm, DV: −7.8 mm. The guide cannula was used for injection of the viral vector and introduction of the optic fiber.

2.3. Viral vectors

Targeting the genetic expression of channelrhodopsin-2 (ChR2) to orexin expressing neurons was achieved by use of a lentivirus carrying the 3.1-kilobase (kb) mouse prepro-hypocretin (Hcrt, which encodes orexin A and orexin B) gene promoter [29]. The Hcrt::ChR2-YFP construct and the Hcrt::YFP control construct were kindly provided by Dr. Luis DeLecea (Department of Psychiatry and Behavioral Sciences at Stanford University). A description of both constructs with the mCherry reporter instead of the YFP can be found in [1]. Briefly, the 3086-base-pair (EcoRI–SacI) mouse Hcrt promoter [29] was used to replace the CaMKIIa promoter in the CaMKIIa::ChR2-YFP lentivirus vector [36]. The Hcrt::YFP control viruses were made by swapping ChR2-YFP with YFP alone. High-titre lentiviruses were produced at the CHOP Viral vector core facility using 293 T cells that were cultured in DMEM growth media containing 10% FBS and 1x P/S antibiotics. The vectors were aliquotted and stored at −80 °C till use. Injection of 4 ul of the high titer virus (approximately 10^9 plaque forming units) directly into hypothalamus was performed the day following surgery. Administering the virus in this manner allowed for injection of freshly thawed virus for all animals at a single time from a single aliquot of virus, which resulted in more consistent ChR2 expression. All rats were unanesthetized and freely moving at time of virus injection. The specificity of lentivirus-mediated expression was tested by dual stain immunocytochemistry for orexinA expression and cFos activation, described in detail below. Peak expression of ChR2 was determined, via fluorescent imaging of YFP signal in freshly sliced tissue, to occur at approximately 4 weeks following virus injection. Therefore, all experiments were begun at 4–5 weeks following injection.

2.4. Experiment 1: validation of construct

2.4.1. Determining the effect of optogenetic stimulation on sleep/wake transitions

The Chr2 construct had not previously been used in rats. In order to validate its functionality, a validation study based on previous work in mice was performed [1]. *In this previous study, optogenetic stimulation of orexin neurons was conducted in sleeping mice and the latency to wakefulness was assessed.* Stimulation of orexin neurons decreased this latency in these mice [1]. In the present experiment, Hcrt::YFP and Hcrt::ChR2-YFP transduced animals ($n = 4$ and 6, respectively) that exhibited behavioral indices of sleep were exposed to optogenetic stimulation of 20 Hz for 10 s at a time every 2 min. *Sleep was assessed by behavioral measures as previous work showed little difference between assessment of sleep through EEG/EMG and high throughput behavioral screening of sleep-wake transitions in mice* [26]. Optogenetic stimulation parameters were chosen based on DeLecea's previous work showing a similar stimulation paradigm increases cFos immunoreactivity in orexin cells in Hcrt::ChR2-mCherry mice compared to the Hcrt::mCherry control animals [1]. *In order to ensure that all rats were tested under similar conditions rats were given a minimum of 4 h of acclimation to the testing room and indwelling optic fiber. Following acclimation, rats were tested in their home cage starting at 1600 h and continued until a minimum of 20 trials was achieved while asleep. Rats were deemed asleep following a minimum of 3 min without any movement as assessed through visual observation.* Rats were monitored for movement while asleep, and the latency to first movement following photostimulation was recorded. Rats then remained undisturbed until they were asleep at which time another trial began. *Because of previous literature showing effects of repeated stress to induce abnormal sleep patterns* [12], we examined the impact of repeated stress on sleep/wake latency. After 20 trials on one day, all rats were then exposed to 4 days of 15 min swim stress each day. On the day following the last swim stress, rats were again monitored for movement during sleep while being exposed to photostimulation, as on the first day.

2.4.2. Examination of optogenetic stimulation effects on cellular activity

To validate the functionality of the ChR2 construct at a cellular level, cFos immunoreactivity in orexin cells was assessed following optogenetic stimulation at 20 Hz for 10 s at a time every minute for 30 min in their home cage. Brains from Hcrt::YFP and Hcrt::ChR2-YFP transduced animals ($n = 10$ and 15 respectively) were collected following photostimulation. Brains were sectioned at 12 μm onto slides and double immunostained for orexinA and cFos. The number of cFos positive/orexinA positive cells was counted and quantified as a percent of total orexin cells on the side of the brain being examined. Briefly, sections were fixed in 10% paraformaldehyde and successively incubated in the following: (1) a goat antiserum to orexinA (1:500, Santa Cruz, sc-8070) in PBST supplemented with 4% normal horse serum (NHS, Sigma) for 3 days at 4 °C; (2) a rabbit antiserum to cFos (1:1,000, Santa Cruz, sc-52) in PBST supplemented with 4% normal horse serum (NHS, Sigma) for 1 day at 4 °C; (3) a donkey anti goat secondary (1:2,000, Abcam, ab6950) and a donkey anti rabbit secondary (1:2000, invitrogen A-21206) both overnight at 4 °C. Finally, the slides were cover slipped with flouromount (Sigma, St Louis, MO). Slides were then visualized on a fluorescence microscope (Leica). Digital images were slightly modified to optimize for image resolution, brightness and contrast in Open Lab software (version 5.5.2, PerkinElmer). Quantification was performed on the virus-injected side and compared to the non-injected side as a control.

2.5. Experiment 2: effects of optogenetically stimulated orexins on behavior in the social interaction test

Naïve Hcrt::YFP and Hcrt::ChR2-YFP transduced animals ($n = 10$ and 15 respectively) were placed in a three-chamber social interaction