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Hypocretinergic neurons are activated in conjunction with goal-oriented survival-related motor behaviors

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ABSTRACT

Hypocretinergic neurons are located in the area of the lateral hypothalamus which is responsible for mediating goal-directed, survival-related behaviors. Consequently, we hypothesize that the hypocretinergic system functions to promote these behaviors including those patterns of somatomotor activation upon which they are based. Further, we hypothesize that the hypocretinergic system is not involved with repetitive motor activities unless they occur in conjunction with the goal-oriented behaviors that are governed by the lateral hypothalamus. In order to determine the veracity of these hypotheses, we examined Fos immunoreactivity (as a marker of neuronal activity) in hypocretinergic neurons in the cat during: a) Exploratory Motor Activity; b) Locomotion without Reward; c) Locomotion with Reward; and d) Wakefulness without Motor Activity. Significantly greater numbers of hypocretinergic neurons expressed *c-fos* when the animals were exploring an unknown environment during Exploratory Motor Activity compared with all other paradigms. In addition, a larger number of Hcrt + Fos + neurons were activated during Locomotion with Reward than during Wakefulness without Motor Activity. Finally, very few hypocretinergic neurons were activated during Locomotion without Reward and Wakefulness without Motor Activity, wherein there was an absence of goal-directed activities. We conclude that the hypocretinergic system does not promote wakefulness *per se* or motor activity *per se* but is responsible for mediating specific goal-oriented behaviors that take place during wakefulness. Accordingly, we suggest that the hypocretinergic system is responsible for controlling the somatomotor system and coordinating its activity with other systems in order to produce successful goal-oriented survival-related behaviors that are controlled by the lateral hypothalamus.

1. Introduction

In 1998, a small group of phenotypically-distinct neurons were discovered in the postero-lateral hypothalamus [1,2]. These neurons were determined to contain the neuropeptides hypocretin 1 and hypocretin 2 (also called orexin 1 and orexin 2), which were later found to function as excitatory neurotransmitters [3]. Hypocretinergic neurons and their receptors have been implicated in a bewildering array of both complementary as well as seemingly contradictory actions and functions ranging from wakefulness to the consumption of food and even the prevention of pain [4–9].

A logical first step in determining the function of any discrete, highly localized group of neurons, such as those that contain hypocretin, is to examine the behaviors and processes that are controlled by the site in the brain wherein they reside. The lateral hypothalamic area, where hypocretinergic neurons are located [1,2,10], is the key brain area that for decades has been identified as being responsible for initiating, coordinating and maintaining goal-oriented survival-type behaviors such as fight, flight and food consumption, among others [11–16]. Hypocretinergic neurons project to all levels of the neuraxis and innervate most central nervous system sites [10]. The activity of the peripheral organs is also influenced by the hypocretins [17]. Hypocretinergic neurons also have the potential to mediate the complex functions that are based in the lateral hypothalamus since they exhibit the morphology of prototypical "command" neurons, which are small groups of highly specialized cells that coordinate and integrate, in a complementary fashion, the activities of a vast number of neural and hormonal systems [18–20]. Accordingly, we hypothesize that the hypocretinergic system functions

Abbreviations: ANOVA, analysis of variance; EEG, electroencephalogram; EMG, electromyogram; EOG, electro-oculogram; NDS, normal donkey serum; PB, phosphate buffer; PBS, phosphate buffer-saline; PGO, ponto–geniculo–occipital; S.E.M., standard error of the mean.

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to promote those goal-directed behaviors that are mediated by the lateral hypothalamus that are critical for the survival of the species.

Previously, utilizing the Fos protein as a marker of neuronal activity, we reported that hypocretinergic neurons are not active during highly aroused states unless the animal is performing motor behaviors [5,21]. Therefore, we concluded that the hypocretinergic system is not engaged simply because an animal is awake, but is rather dedicated to supporting certain motor behaviors that occur during the waking state. To further explore these findings, in the present study we compared Fos immunoreactivity in hypocretinergic neurons in cats, when they were awake but not exhibiting motor activity, during motor behaviors that were goal-directed, and in conjunction with other behaviors that were not goal-directed. We found that hypocretinergic neurons do not express *c-fos* during wakefulness in the absence of motor activity or during repetitive motor activities, but that they are active during specific goal-directed motor behaviors.

2. Material and methods

2.1. Experimental animals

Fifteen adult male cats were utilized in this study. All animals, which were determined to be in good health by veterinarians, were housed under normal laboratory conditions (21–23 °C, 12 h day–night cycle, lights on at 7:00 AM). Experimental procedures were conducted in accordance with the Guide to the Care and Use of Laboratory Animals (8th edition, National Academy Press, Washington D. C., 2010). Adequate measures were taken to minimize pain, discomfort or stress of the animals. In addition, all efforts were made in order to use the minimal number of animals necessary to produce reliable scientific data.

2.2. Surgical procedures

Six cats (corresponding to the Wakefulness without Motor Activity group, see below) were implanted, under general anesthesia, with electrodes for recording the electroencephalogram (EEG), electromyogram (EMG), electro-oculogram (EOG) and ponto–geniculo–occipital (PGO) waves in order to monitor the states of sleep and wakefulness. Prior to being anesthetized, each cat was pre-medicated with Xylazine® (2.2 mg/kg, i.m.), atropine (0.04 mg/kg, i.m.) and antibiotics (Tribrisren®, 30 mg/Kg, i.m.). Anesthesia, which was first induced with ketamine (15 mg/kg, i.m.), was maintained with a gas mixture of isoflurane in oxygen (1–3%). The head was positioned in a stereotaxic frame and the skull was exposed. Stainless steel screw electrodes were placed in the frontal and parietal bones to record EEG activity and in the orbital portion of the frontal bone to record eye movements. Bipolar electrodes were implanted in both lateral geniculate nuclei to monitor PGO waves. These permanently implanted electrodes were connected to a Winchester plug, which together with a chronic head-restraining device, were bonded to the skull with acrylic cement. At the end of the surgical procedures, an analgesic was administered (Buprenex®, 0.01 mg/kg, i.m.). Incision margins were kept clean and a topical antibiotic was administered for a period of 5 days. In previous reports we have employed the same surgical procedures [22–24].

2.3. Experimental groups

All animals had free access to water and food until 1 h prior to being placed in different experimental situations, in which they exhibited: Exploratory Motor Activity, Locomotion without Reward, Locomotion with Reward, or Wakefulness without Motor Activity (see Results section).

Experimental sessions were conducted between 10:00 and 15:00 hours at a controlled ambient temperature of 21–23 °C. Each session was 90 to 120 min in duration in order to provide for the maximum expression of the Fos protein [25–27]. We have previously demonstrated that this time period is appropriate for the expression of Fos in the cat under conditions that were similar to those present in the current study [23,28]. In selected experiments, the animals were fitted with an Actiwatch (actigraph) device (Minimitter) [21,29], which is a removable collar apparatus that records motor activity. All animals were euthanized with an overdose of pentobarbital (50 mg/kg) after the conclusion of the experimental sessions.

2.4. Immunohistochemical procedures

The following immunohistochemical procedures, which were employed for the detection of Fos and hypocretin-2, have been used in previous studies from our laboratory [5,21,23]. Briefly, at the termination of the experimental paradigms, the animals were perfused with 1 l of heparinized saline followed by 1 1/2 l of a solution of 4% paraformaldehyde, 15% saturated picric acid and 0.5% of glutaraldehyde in phosphate buffer (PB, 0.1 M, pH 7.4). Subsequently, they were perfused with 1.2 l of the same solution with 10% sucrose. The brain was then removed and immersed for 24 hours in 2% paraformaldehyde, 15% saturated picric acid and 10% sucrose in PB. Following postfixation, the tissue was kept for three days in a solution of sucrose (25%) and sodium azide (0.1%) in PB. Thereafter, the brain was frozen and serially sectioned at 20 μm using a Reichert–Jung cryostat. The sections were stored in a solution of 0.1% sodium azide in phosphate–buffer–saline (PBS, 0.1 M).

Immunostaining was first carried out to detect the Fos protein. For this purpose, free-floating sections of the hypothalamus were incubated overnight in a rabbit polyclonal Fos antiserum (Fos Ab5; Oncogene Research Products) at a dilution of 1:40,000 in PBS. The sections were rinsed four times in PBS during a period of 30 min and then incubated for 90 min in a solution of biotinylated donkey anti-rabbit immunoglobulin G (1:700). Subsequently, the sections were incubated with the ABC complex (Vector ABC Elite kit, 1:500) for 60 min. After another rinsing, the tissue was reacted for 10–20 min with 0.6% nickel ammonium sulfate, 0.02% diaminobenzidine tetrahydrochloride (Sigma) and 0.015% hydrogen peroxide in 50 ml of 50 mM tris buffer, pH 7.5.

Antibodies directed against hypocretin-2 were employed because hypocretin-1 is co-localized with hypocretin-2 in hypocretinergic neurons in the cat [30]. Accordingly, sections that were previously treated for Fos were then incubated overnight with a hypocretin-2 antibody (1:15,000) and normal donkey serum (NDS; 3%). The polyclonal rabbit antibody (Phoenix Pharmaceuticals) raised against human hypocretin-2 (orexin B) was employed; it has been documented to exhibit 100% cross-reactivity with human hypocretin antigen and no cross-reactivity with other related peptides (data supplied by Phoenix Pharmaceuticals). The sections were subsequently rinsed and incubated for 90 min with biotinylated donkey anti-rabbit antibody (1:300) plus NDS. After another rinse, the tissue was incubated with the ABC complex (1:200) for 60 min and then exposed to diaminobenzidine and hydrogen peroxide (without nickel enhancement). For all procedures, four final rinses with 0.01 M PBS preceded the mounting of sections on slides.

Sections of the hypothalamus were counterstained with Pyronin-y in order to identify the cytoarchitecture of this region. Successful pre-absorption controls were performed in our laboratory using hypocretin peptides prior to immunostaining sections.

2.5. Data analysis

2.5.1. Behavioral data

Polysomnographic recordings of EEG, EMG, EOG and PGO activity were used to document the states of sleep and wakefulness; these recordings were monitored on-line and stored using Superscope®