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Intermittent but not sustained hypoxia activates orexin-containing neurons in mice

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ABSTRACT

Hypothalamic orexin-containing neurons are activated by CO_2 and contribute to hypercapnic ventilatory activation. However, their role in oxygen-related regulation of breathing is not well defined. In this study, we examined whether an experimental model mimicking apnea-induced repetitive hypoxemia (intermittent hypoxia [IH]) activates orexin-containing neurons. Mice were exposed to IH (5×5 min at 10% O_2), intermittent hyperoxia (IO; 5×5 min at 50% O_2), sustained hypoxia (SH; 25 min at 10% O_2), or sham stimulation. Their brains were examined using double immunohistochemical staining for orexin and c-Fos. The results indicated that IH ($25.8 \pm 3.0\%$), but not SH ($9.0 \pm 1.5\%$) activated orexin-containing neurons when compared to IO ($5.5 \pm 0.6\%$) and sham stimulation ($5.9 \pm 1.4\%$). These results correlate with those of our previous work showing that IH-induced respiratory long-term facilitation is dependent on orexin-containing neurons. Taken together, orexin contributes to repetitive hypoxia-induced respiratory activation and the hypoxic activation of orexin-containing neurons is pattern dependent.

1. Introduction

Orexin (Sakurai et al., 1998) is a neuropeptide also known as hypocretin (de Lecea et al., 1998). It is a hypothalamic neuropeptide best known as a wake period stabilizer (Sakurai, 2007), as injection of orexin into the brain prolongs the wake period and shortens sleep. Deficiencies in orexin cause narcolepsy characterized by sudden cataplectic attacks and sleep/wake fragmentation (Thannickal et al., 2000). The activity of orexin-containing neurons is minimal when animals are sleeping and increases in parallel with attention level increases from quiet wake to active wake periods during exploration or stress (Mileykovskiy et al., 2005). Activity of orexin-containing neurons is also affected by circadian rhythmicity (Estabrooke et al., 2001; Saper et al., 2005). Orexin-containing cell bodies are found exclusively in the lateral hypothalamus (LH), perifornical area (PeF), and the dorsomedial hypothalamus (DMH), whereas orexin-containing fibers and terminals are widely distributed in almost all brain regions (Sakurai, 2007). This anatomic feature enables orexin to contribute to the control of multiple physiological functions including sleep–wake cycling, feeding behavior, energy homeostasis, motivation, and regulation of the respiratory, autonomic, and neuroendocrine systems (Sakurai, 2007). Specific respiratory-related sites that receive orexinergic innervation are the nucleus tractus solitarius, pre-Bötzinger complex, and the retrotrapezoid, hypoglossal, raphe, parabracheal, and phrenic nuclei. Therefore, orexin affects respiratory rhythm and activity of neurons controlling pump and airway valve muscles (see Kuwaki, 2008 for review).

The contribution of orexin to respiratory regulation seems to occur in a vigilance state-dependent manner. We have previously shown that hypercapnia-induced ventilatory activation was attenuated in orexin knockout mice as compared to their wild-type littermates when the animals were in a wakeful period, but not during sleep (Nakamura et al., 2007). However, basal ventilation remained normal irrespective of the vigilance state. In addition, respiratory activation as a component of the defense response against stress also requires orexin activity (Iigaya et al., 2012; Kayaba et al., 2003; Zhang et al., 2006). Nattie and colleagues showed that

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activity of an orexin receptor antagonist on hypercapnic ventilatory activation was prominent at night, which is the active period for rodents (Dias et al., 2009; Dias et al., 2010). In support for these observations, studies have shown that orexin-containing neurons were activated by carbon dioxide in vitro (Williams et al., 2007) and in vivo when animals were awake (Sunanaga et al., 2009).

On the other hand, the potential role of orexin in hypoxia-induced ventilatory activation – another important regulatory mechanism for respiration – has been sparsely studied except for our previous studies showing the essential role of orexin-containing neurons in repetitive intermittent hypoxia (IH)-induced respiratory long-term facilitation (LTF) (Terada et al., 2008; Toyama et al., 2009). IH, a model of sleep apnea-induced hypoxemia, induces long lasting (>1 h) augmentation of respiratory motor output that occurs even after the cessation of hypoxic stimuli (Feldman et al., 2003; Mitchell and Johnson, 2003; Powell et al., 1998). This LTF phenomenon relies on serotonin-containing neurons in the raphe nuclei that receive dense projections from the orexin-containing neurons in the hypothalamus (Kuwaki, 2008). LTF is pattern sensitive in such a way that occurs after repetitive IH (>3 times) but not after sustained hypoxia (SH) (Feldman et al., 2003; Millhorn et al., 1980).

The pattern sensitive nature of LTF resembles the behavioral state-dependent nature of the orexinergic control of respiration. Actually, absence of orexin results in attenuation of LTF (Terada et al., 2008; Toyama et al., 2009). Nevertheless, it is currently unknown whether orexin-containing neurons are activated by IH/SH. Therefore the goal of this study was to determine whether IH and SH activate orexin-containing neurons using immunohistochemistry. We also tested whether intermittent hyperoxia (IO) affected orexin-containing neuron activity, since we identified that IH activated orexin-containing neurons. These results were not observed with SH, therefore, we hypothesized that repetitive alterations in oxygen and not decreased oxygen may affect neuronal activity.

2. Methods

2.1. Animals

Experiments were performed using C57BL/6 mice (12–20 weeks old, Japan SLC, Shizuoka, Japan). All mice were males to avoid possible effect of menstrual cycle. The mice were housed in plastic cages in a room maintained at 23–25 °C with the lights on at 7:00 AM and off at 7:00 PM. The mice were provided food and water ad libitum. All experimental procedures were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Use Committee of Kagoshima University.

2.2. Protocols

The animals were divided into 4 groups including 5 mice per group. Prior to each experiment, the mice were acclimatized by placing each unanesthetized mouse in a plastic chamber (750 mL) that allowed free movement with an airflow of 2 L/min for >2 h. Following acclimatization, the first group of animals was exposed to SH for 25 min (10% O_2; 1:1 mixture of room air and N_2). The second group was exposed to IH 5 times (with each session including 5 min of 10% O_2 separated by 5 min of room air). The third group was exposed to IO 5 times (with each session including 5 min of 50% O_2 separated by 5 min of room air). The last group served as a control and received sham stimulation 9 times (which included 5 min of room air separated by a transient cessation of gas flow) to mimic the flow change between 10% and 50% O_2 and room air in the IH/IO protocols. The concentration of O_2 in the chamber was monitored continuously by an O_2 sensor (model JKO-25LJ II CM, JIKCO, Tokyo, Japan) at the outlet port of the chamber. Stimulation was applied during the afternoon (Zetigeber time (ZT) 6–9) when the activity levels and wakefulness of the mice were minimal (Kayaba et al., 2003) and the activity of orexin-containing neurons was minimal (Estabrooke et al., 2001). Wakefulness of the animals was confirmed by observation of their behavior and we noted that the mice were sleeping at the start of hypoxic stimulation.

One hour post-stimulation, the mice were deeply anesthetized by an intraperitoneal injection of urethane (1.8 g/kg) with heparin sodium and transcardially perfused with 0.01 M phosphate-buffered saline (PBS) followed by a fixative solution containing 4% paraformaldehyde in PBS. The brains were excised and post-fixed in the same fixative solution for 24 h at 4 °C.

To avoid the potential confounding factor of remaining odor from the previous animal, the chamber was thoroughly cleaned before the next experiment.

2.3. Immunohistochemistry

Serial transverse sections (40 μm) were cut from the brain tissue including the hypothalamus using a vibratome (model Zero1, DOSAK EM, Kyoto, Japan). Every fourth section was collected in PBS and processed as a free-floating section on a shaking table at room temperature. The sections were incubated sequentially with the following protocol: first, the sections were incubated with PBS containing 0.3% Triton-X and 2% normal horse serum for 30 min; second, the sections were incubated with rabbit anti-c-Fos antiserum (1/1000, Calbiochem, Darmstadt, Germany) for 90 min followed by biotinylated donkey anti-rabbit IgG antibody (1/250, Jackson Immunoresearch, West Grove, PA, USA) for 90 min; third, the sections were incubated with goat anti-orexin antiserum (1/100, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 90 min; and finally the tissue was incubated with Alexa Fluor 488 streptavidin conjugate (1/200, Molecular Probes, Eugene, OR, USA) and CF568-labeled donkey anti-goat IgG antibody (1/200, Biotium, Hayward, CA) for 90 min in a dark box. The sections were then mounted on a glass slide and examined using a fluorescence microscope (BZ8000, Keyence, Osaka, Japan). The images were recorded at ×100 with a 24-bit digital camera (4080 × 3072 pixel). Specificity of antibodies was determined using negative control sections incubated without primary or secondary antibody for each experiment. We did not detect a signal in the negative controls.

2.4. Cell counting and statistics

The number of single-labeled (orexin) or double-labeled (orexin plus c-Fos) cells was determined for the stained hypothalamus sections in a blinded manner to the treatment. The cells were counted in the medial and lateral hypothalamic areas defined by a vertical line just lateral to the fornix (Fig. 1). This enabled assessment of two distinct populations of orexin-containing neurons suggested as having different physiological roles (Harris and Aston-Jones, 2006; Sunanaga et al., 2009; Zhang et al., 2009).

Data were analyzed by a nonparametric ANOVA, Kruskal–Wallis test, and Dunn's post hoc test. Comparison between the numbers of orexin-containing neurons in the medial hypothalamic area and that in the lateral hypothalamic area was performed using a nonparametric-paired test, Wilcoxon matched pairs test. Data were presented as mean ± SEM. Differences were considered significant at $p < 0.05$.