Effects of gamma-aminobutyric acid A-receptor antagonist on sleep-wakefulness cycles following lesion to the ventrolateral preoptic area in rats

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Abstract

BACKGROUND: Neurons expressing gamma-aminobutyric acid (GABA) play an important role in the regulation of wakefulness to sleep, as well as the maintenance of sleep. However, the role of GABAergic neurons in the tuberomammillary nucleus (TMn), with regard to the sleep-wakefulness cycle, is poorly understood.

OBJECTIVE: To investigate the effects of GABAergic neurons in the TMn on the sleep-wakefulness cycle.

DESIGN, TIME AND SETTING: Randomized controlled study, performed at the Laboratory of Neurobiology, Department of Anatomy, Histology and Embryology, School of Basic Medical Sciences, Lanzhou University from July 2007 to February 2008.

MATERIALS: Fifteen healthy, adult, male, Sprague Dawley rats were randomly divided into three groups (n = 5): control, ventrolateral preoptic area (VLPO) lesion, and VLPO lesion plus GABAA receptor antagonist-treated. Ibotenic acid and bicuculline were provided by Sigma (St. Louis, USA).

METHODS: Four electroencephalogram screw electrodes were implanted into the skull at a frontal region (two) and parietal bones (two) on each side. Three flexible electromyogram wire electrodes were placed into the nuchal muscles. On day 8, a fine glass micropipette (10–20 mm tip diameter) containing ibotenic acid solution (10 nmol/L) was injected into the VLPO in both hemispheres following bone wax removal under anesthesia. One week after the second surgery, sleep-wakefulness states were recorded in rats from the VLPO lesion group. On day 10 after VLPO lesion, bicuculline (10 nmol/L), a GABAA-receptor antagonist, was microinjected into the TMn and sleep-wakefulness states were recorded for 24 hours.

MAIN OUTCOME MEASURES: Duration of the sleep-wakefulness cycle in each group using a Data acquisition unit (Micro1 401 mk2) and Data collection software (Spike II).

RESULTS: VLPO lesion induced an increased duration of wakefulness (W, 13.17%) and light slow-wave sleep (SWS1, 28.9%), respectively. Deep slow-wave sleep (SWS2, 43.74%) and paradoxical sleep (PS, 44.07%) were respectively decreased for 24 hours at day 9 post-lesion, compared with pre-lesion (P < 0.01). Microinjection of bicuculline into the TMn following VLPO lesion at 10:00 am on the 10th day elicited a wake state for 40–55 minutes, with a latency of 15 minutes. However, 24-hour sleep-wake states demonstrated that the ratio of W and SWS1 were increased by 12.61% (P < 0.01) and 50.97% (P < 0.01), respectively. In addition, SWS2 and PS were decreased by 68.08% (P < 0.01) and 39.92% (P < 0.05), respectively, compared with prior to VLPO lesion.

CONCLUSION: The evidence of decreased deep slow-wave sleep, which was induced by VLPO lesion, suggested that GABAergic neurons in the VLPO play an important role in maintaining sleep. Bicuculline microinjection into the TMn, following VLPO lesion, elicited wakefulness and sleep depression for 50 minutes, with contrary increased light slow-wave sleep for 24 hours, which suggested that GABAergic neurons in the TMn play a role in sleep drive (sleepiness) via local circuit to directly inhibit histaminergic neurons.

Key Words: GABAergic neuron; GABAA-receptor antagonist; histaminergic neuron; neurotoxic lesion; tuberomammillary nucleus; ventrolateral preoptic area
INTRODUCTION

Neurons expressing gamma-aminobutyric acid (GABA) have been shown to play an important role in the regulation of wakefulness to sleep[1-9]. GABAergic neurons in the hypothalamus are not only located in the ventrolateral preoptic area (VLPO), but also in the tuberomammillary nucleus (TMn) of the posterior hypothalamus[4-10]. The role of GABAergic VLPO neurons in sleep-wake regulation has been intensively studied during the past decades. However, the role of GABAergic neurons in the TMn remains poorly understood. It is well known that histaminergic (HA) neurons are exclusively located in the TMn and induce neocortical activation of wakefulness[11-17]. Descending projection from the raphe nucleus (DRN), and locus coeruleus (LC)[1-8, 21-22]. GABAergic neurons[18-19, 24-26], and receives GABAergic terminals from the TMn[27-28]. Neuronal structural characteristics in the TMn in the posterior hypothalamus provide input to the VLPO[27-28]. Neuronal structural characteristics in the posterior hypothalamus, as well as reciprocal projections between the VLPO and the TMn of the posterior hypothalamus, suggest that the posterior hypothalamus plays an important role in the regulation of sleep and wakefulness. However, the role of the GABAergic neurons was not determined in the present study. Approximately 80% of cells in the VLPO are GABAergic neurons[20]. Therefore, the present results suggest that the GABAergic neurons are responsible for the role of the VLPO in the regulation of sleep and wakefulness, rather than the TMn. However, the role of the GABAergic neurons in the TMn cannot be disregarded, because studies have shown that muscimol, a potent GABA\textsubscript{A} receptor agonist, microinjection into the TMn induces pronounced and long-lasting increased slow-wave sleep (SWS)[27-28]. Recordings from individual VLPO neurons in hypothalamic slices has shown that these neurons are inhibited by noradrenaline and 5-HT[9]. No responses to histamine were recorded, but TMn neurons also express GABA and galanin, which might serve to inhibit the VLPO[18]. These results suggest that the HA might interact with GABAergic neurons in the TMn, and conduct impulses to the VLPO and other areas.

The purpose of the present study was to investigate the effect of GABAergic neurons in the TMn on the sleep-wakefulness cycle. A small dose of ibotenic acid, a cell-specific neurotoxin[29], was injected into the VLPO region to disturb transmission from the VLPO to the TMn. In addition, bicuculline, a GABA\textsubscript{A} receptor antagonist, was microinjected into the TMn, in order to determine whether the GABAergic neurons in TMn participate in sleep regulation.

MATERIALS AND METHODS

Materials
Fifteen pathogen-free, adult, male, Sprague Dawley rats, weighing 250–300 g, were purchased from the Experimental Animal Center of Lanzhou University (Certification number: 14-0006). All rats were individually housed and provided free access to food and water. The rats were housed under controlled conditions (interval 12-hour light/dark cycle) in an isolated, ventilated chamber maintained at 22–24 °C and 50% humidity. The experimental animals were sacrificed according to Instructions for the Care of Laboratory Animals published by the Ministry of Science and Technology in 2006[30]. All rats were randomly divided into three groups: control, model, and treatment, with five rats in each group.

<table>
<thead>
<tr>
<th>Reagent and equipment</th>
<th>Source</th>
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<tbody>
<tr>
<td>Ibotenic acid (FW158.13) and bicuculline (FW 367.36)</td>
<td>Sigma (St. Louis, USA)</td>
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<tr>
<td>EEG-5208 10 channels EEG machine</td>
<td>Nihon Kohden Corporation (Tokyo, Japan)</td>
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<td>Data acquisition unit (Micro 1401 mk2)</td>
<td>CED, UK</td>
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<tr>
<td>Data collection software (Spike 2)</td>
<td>CED, UK</td>
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Methods

Electroencephalogram electromyogram implantation, sleep recording, and scoring
Rats were anesthetized with pentobarbital (2% in saline, 35 mg/kg, i.p.), prepared for aseptic surgery, and secured in a SR-6R stereotaxic frame (Narishige, Tokyo, Japan) with a tooth bar set at -3.3 mm. Four stainless steel, cortical electroencephalogram (EEG) electrodes were inserted into the dura through two pairs of holes made in the skull, and were located, respectively, in the frontal (1 mm lateral and anterior to the bregma) and occipital (1 mm lateral to the midline and 1 mm anterior to the lambda) cortices. Three flexible, wire, electromyogram (EMG) electrodes were placed under the nuchal muscles. The dura was then gently disrupted at the hole, and two 23-gauge stainless steel, guide cannulas were inserted to 2.0 mm above the TMn (anteroposterior, -4.3 mm; dorsoventral, -7.4 mm; and mediolateral, ±1.3 mm with reference to the bregma), according to coordinates from the atlas of Paxinos and Watson[31]. EEG/EMG electrodes were connected to a pedestal socket. Dental cement was then used to affix...
all leads and cannulas to the skull. A small area (0.50 cm) surrounding the bregma was left without dental cement and filled with removable bone wax.

**Ibotenic acid injection**

Animals were anesthetized with pentobarbital, the bone wax was removed, and a fine, glass micropipette (10–20 mm tip diameter) was stereotactically inserted into the VLPO area in each hemisphere. The coordinates used for the VLPO (anteroposterior, −0.6 mm; dorsoventral, −8.5 mm; and mediolateral, ±1.2 mm with reference to the bregma) were according to coordinates from the atlas of Paxinos and Watson[26], with the tooth bar to −3.3 mm. Ibotenic acid solution (1 nmol/L) was injected. After 5 minutes, the pipette was slowly withdrawn. The wound was treated with terramycin ointment, filled with sterile bone wax, and closed with wound clips.

**Experimental protocol**

After a one-week recovery period, 24-hour basic wake-sleep states were recorded in animals of “before lesion”. One week after the second surgery, sleep-wakefulness states were recorded in rats from the VLPO lesion group for 2 weeks. Drugs were microinjected into both TMns between 10:00 and 10:20 on day 10 after VLPO lesion, and polysomnogram (PSGs) were recorded at 8:00 am for 24 hours. After a one-week recovery period, the animals were adapted to the EEG/EMG recording apparatus for 24 hours prior to recordings. EEG and EMG signals were amplified by a EEG-5208 10-channel EEG machine, then digitized (sampling rate of 128 Hz) and stored on a computer using data collection software. Sleep-wake states were manually scored in 30-second epochs, according to typical criteria, by an observer who was unaware of the experimental design. Wakefulness (W) was identified by the presence of desynchronized-EEG and high-EMG activity. SWS was identified by the presence of a high-amplitude slow-wave EEG and presence of desynchronized-EEG and high-EMG activity. SWS was identified by the presence of desynchronized-EEG and high-EMG activity. SWS was identified by the presence of desynchronized-EEG and high-EMG activity. SWS was identified by the presence of desynchronized-EEG and high-EMG activity.

**Main outcome measures**

The duration sleep-wakefulness cycles in each group.

**Statistical analysis**

Statistical analysis was performed by Xin Zhang with SPSS version 11.5 for windows. The values were presented as Mean ± S.E.M. The data were analyzed with within-subjects analysis of variance (ANOVA) and the Dunnett’s t-test to determine significant differences. P < 0.05 was considered significant.

**RESULTS**

**Quantitative analysis of subjects**

All rats were involved in the final analysis without any loss.

**Effect of VLPO lesion on the ratio of sleep-wakefulness**

After VLPO lesion with ibotenic acid (n = 5), the ratio during 24 hours of W and SWS1 were respectively increased by 13.17% (P < 0.01) and 28.9% (P < 0.01). In addition, SWS2 and PS were respectively decreased by 43.74% (P < 0.01) and 44.07% (P < 0.01) on the 9th day, compared with the pre-lesion group. These results indicated that ibotenic acid lesions could inhibit the sleep-maintaining effect of GABA in the VLPO (Figures 1–3).

**Effects of bicuculline microinjection into the TMn on the sleep-wakefulness cycle following VLPO lesion**

TMn microinjection of bicuculline into VLPO lesion animals at 10:00 am on day 10 elicited a wake period for 40–55 minutes, with a latency of 15 minutes. However, 24-hour sleep-wake states demonstrated that the ratio of W and SWS1 were increased by 12.61% (P < 0.01) and 50.97% (P < 0.01) respectively when compared with the pre-lesion group. While SWS2 and PS were decreased by 68.08% (P < 0.01) and 39.92% (P < 0.05) respectively when compared with the pre-lesion group (Figures 1–3).

**Figure 1** 24-hour curvilinear graphs show light slow-wave sleep (SWS1) state prior to ventrolateral preoptic area (VLPO) lesion in control, at day 9 post-VLPO lesion, and VLPO lesion animals microinjected with bicuculline (10 nmol/L/0.5 μL) into tuberomammillary nucleus at 10:00 on the day 10.
DISCUSSION

The cell-specific excitotoxin ibotenic acid, which does not destroy the fibers of passage, was used to produce a VLPO lesion to determine whether these neurons are necessary for producing sleep. Results confirmed the role of VLPO neurons in the promotion of sleep. Furthermore, the data demonstrated that VLPO lesion specifically reduced SWS2, which suggested that GABAergic neurons in the VLPO play an important role in maintaining sleep. However, the increased ratio of SWS1 indicated that sleep-driving neurons originated from GABAergic neurons in the TMn, not from the VLPO. Rats with VLPO lesion displayed much more wakefulness, as well as an increased homeostatic drive for sleep [32]. Therefore, these rats fell asleep more frequently, but switched from SWS2 to SWS1 more frequently. In addition, the SWS2 state was shorter following VLPO lesions.

Neurons of the HAergic TMn express the neurotransmitter GABA [18] and project to the VLPO [27-28]. Approximately 80% of VLPO neurons contain the inhibitory neurotransmitter GABA, and they provide TMn GABAergic innervation to other arousal systems [20]. Recordings from individual VLPO neurons in hypothalamic slices demonstrated that they are inhibited by noradrenaline and 5-HT [9]. However, although responses to histamine were not recorded, TMn neurons were shown to express GABA and galanin, which could inhibit the VLPO [19]. These results suggest that the HA might interact with GABAergic neurons in the TMn, and also conduct impulses to the VLPO and other regions. An additional microinjection of bicuculline, a GABAA-receptor antagonist, into the TMn following VLPO lesion directly elicited a reversible waking state with a latency of 15 minutes. However, the tendency of increased SWS1 states and decreased SWS2 states could be altered, especially during the daytime. These data suggested that local GABAergic neurons acted directly on HAergic neurons to induce a sleep onset/sleep drive from wake to sleep. The presumed circuitry between GABAergic and HAergic neurons in the TMn was short and direct for initial altered sleep-wakefulness. Moreover, the bicuculline-treated rats, with VLPO lesion, displayed a reversible waking state, and altered from wake to increased SWS1 and decreased SWS2. Defective sleep was greater in the bicuculline-treated group with VLPO lesion, compared with the saline-treated group with VLPO lesion.

In conclusion, deep slow-wave sleep was significantly decreased due to VLPO lesion, which suggests that GABAergic neurons in the VLPO play an important role in sleep maintenance. Microinjections of bicuculline into the TMn, following VLPO lesion, elicited wakefulness and sleep depression for 50 minutes. However, in this study light slow-wave sleep was significantly increased for 24 hours, which suggested that GABAergic neurons in the TMn play a role in sleep drive (sleepiness) via local circuitry by directly inhibiting histaminergic neurons.
REFERENCES


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