Attenuated defense response and low basal blood pressure in orexin knockout mice

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Kayaba, Yuji, Akira Nakamura, Yoshitoshi Kasuya, Takashi Ohuchi, Masashi Yanagisawa, Issei Komuro, Yasuichiro Fukuda, and Tomoyuki Kuwaki. Attenuated defense response and low basal blood pressure in orexin knockout mice. Am J Physiol Regul Integr Comp Physiol 285: R581–R593, 2003. First published May 15, 2003; 10.1152/ajpregu.00671.2002.—The perifornical area of the hypothalamus has been known as the center for the defense response, or “fight or flight” response, which is characterized by a concomitant rise in arterial blood pressure (AP), heart rate (HR), and respiratory frequency (RF). We examined whether orexin, a recently identified hypothalamic neuropeptide, contributes to the defense response and basal cardiovascular regulation using orexin knockout mice. Microinjection of a GABA-A receptor antagonist, bicuculline methiodide (0.1–1 mM in 20 nl), to the perifornical area in urethane-anesthetized wild-type mice elicited dose-dependent increases in AP, HR, and RF. Although similar changes were observed in orexin knockout mice, intensities were smaller and duration was shorter than those in wild-type mice. Moreover, in an awake and freely moving condition, telemeter-indwelling orexin knockout mice showed diminished cardiovascular and behavioral responses to emotional stress in the resident-intruder test. We also found that basal AP in orexin knockout mice was significantly lower in both anesthetized (117±8 mmHg in wild type and 92±3 in knockout) and conscious (125±6 mmHg in wild type and 109±2 in knockout) conditions. α-Adrenergic blockade with prazosin or ganglion blockade with hexamethonium canceled the difference in basal AP. HR and cardiac contractile parameters by echocardiography did not differ between the two strains of mice. These results indicate lower sympathetic vasoconstrictor tone in knockout mice. The present study suggests that orexin-containing neurons in the perifornical area play a role as one of the efferent pathways of defense response and also operate as a regulator of AP at basal condition by activating sympathetic outflow.

hypothalamus; stress; respiration; sympathetic nervous system; circadian rhythm.

OREXIN A and B, also known as hypocretin 1 and 2, are recently identified neuropeptides that consist of 33 and 28 amino acids, respectively (10, 46). They are proteolytically derived from the same precursor peptide (pre-pro-orexin) and exert a variety of functions by acting on orexin receptor type 1 and/or type 2. Orexin-containing neuron cell bodies are located exclusively in the lateral and dorsal hypothalamic areas and their axons diffusely innervate almost the entire central nervous system (6, 9, 10, 38, 39, 44, 46). This anatomic feature establishes the bases that orexin contributes to multiple physiological functions, including feeding behavior (46), energy homeostasis (46, 54), sleep-wake cycle (5), and regulation of the autonomic and neuroendocrine systems (9, 22, 44, 54).

Several laboratories have proposed a possible contribution of orexin in cardiovascular regulation by observing the effects of exogenously administered orexins. Orexins on intracerebroventricular injection increased arterial blood pressure (AP), heart rate (HR) (47), renal sympathetic nerve activity, and plasma catecholamines (50) in conscious, unrestrained rats. Intrathecal administration of orexins increased AP and HR (3). Microinjection of orexin A to the rostral ventrolateral medulla (RVLM) increased AP and HR in anesthetized rats (7) and awake rats (36). However, there is no report to date on whether the same is true for intrinsic orexin.

On the other hand, the perifornical area of the hypothalamus or dorsomedial hypothalamus, a region of the brain with the highest density of orexin-containing neurons (9, 10, 44, 46), has been known as the center for defense response and is sometimes called the defense area (29). Defense response, which is also known as “fight-or-flight” response, is characterized by a coordinated rise in AP, HR, respiratory frequency (RF), and resistance in most vascular beds along with a fall in resistances in airway and blood vessels in the skeletal muscles when an animal encounters stressors. A pioneer work by Hess (23) showed that electrical stimulation of the posterior hypothalamus in cats elicited behavioral rage, along with the specific autonomic responses, that was termed the “defense response.” Although some reports using chemical stimulation with
excitatory amino acids (e.g., glutamate) had shown no effect or even a depressor effect (19, 25), later it was shown that negative results might have been caused by the stimulation of specific subregions in the posterior hypothalamus and/or dosage of drugs (14). Actually, the perifornical area was the most reliable region to elicit the cardiovascular defense response (29), and an overdose of excitatory amino acids sometimes inhibited neuronal activity, an effect known as excitation block phenomenon (34). Disinhibition with a GABA-A receptor antagonist, biccuculline methiodide, of the perifornical area reliably and dose dependently induced the defense response (13). Moreover, injection of a GABA agonist to the defense area inhibited the stress-induced rise in AP and HR (35).

There is only limited information about neurotransmitter(s) that subserve efferent pathways of the defense responses. Namely, glutamate was proposed to mediate at least the cardiovascular component of the defense response (52). However, there is no report on the molecular basis of the defense response underlying its multifaceted nature such as concomitant and coordinated changes in cardiovascular, respiratory, and behavioral parameters. We hypothesized that intrinsic orexin, synthesized in the perifornical area, may participate in the efferent pathway of the defense response and thought that knockout mice may be useful to test the hypothesis.

Prepro-orexin knockout mice, which completely lack both orexin A and orexin B, have been recently established (5). They exhibited a phenotype strikingly similar to human narcolepsy patients, such as behavioral arrest resembling cataplectic attack, sleep-onset rapid eye movement sleep, and diminished awake time in the dark period. This murine model and the canine model (11) and may be a part of defense response. EEG cated as one of the functions of the perifornical lateral hypothalamus (11) and may be a part of defense response. In fact, human narcolepsy seems to be an autoimmune disease, resulting in loss of orexin-containing neurons in the hypothalamus (53). On the other hand, there are only a few reports examining autonomic regulation in human narcolepsy (see DISCUSSION). Cardiovascular and autonomic phenotypes in orexin knockout mice have not been examined.

The aim of this study was to test our hypothesis by 1) identifying hypothalamic subregion(s) in mice suitable to elicit the defense response and by 2) comparing the effect of hypothalamic stimulation between orexin null mutated knockout mice and wild-type mice. Possible involvement of orexin in the defense response was further clarified by 3) telemetric measurement of AP and HR during socioemotional stress of the resident-intruder test. In the course of the experiments, we found lower basal AP in orexin knockout mice. Therefore, an additional aim of this study was to examine possible involvement of intrinsic orexin in basal cardiovascular regulation by 4) comparing AP and HR between mutant and wild-type mice in unanesthetized freely moving condition with or without pharmacological perturbations.

**MATERIALS AND METHODS**

**Animals.** Prepro-orexin mutated mice of genetic background of a mixture of 129Sv and C57BL/6 were generated as reported previously (5). They were maintained in heterozygotes and crossed to obtain null mutants and wild-type littermates. Genotype of orexin knockout mice was identified by PCR on DNA extracted form a tail biopsy in a similar manner as had been reported except for primer sequences. We used a 5′ primer, GAC CTA TCA GGA CAT GTC GCC and a 3′ primer, TCA CCC CCT TGG GAT AGC CTT GCC for the mutant allele and a 5′ primer, GAC GAC GTC AGA CTT GGG with the same 3′ primer to identify the wild-type allele. Mice in this study were 18- to 40-wk-old male orexin null mutated mice and wild-type mice. Heterozygotes were not used. All mice were housed in plastic cages in a room maintained at 23–25°C with lights on at 7:00 AM and off at 7:00 PM. Mice had food and water available ad libitum. All animal procedures conformed to the “Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences” recommended by the Physiological Society of Japan.

**Measurement of cardiorespiratory parameters and electroencephalogram in anesthetized mice.** Mice were anesthetized with intraperitoneal injection of urethane (1.1 g/kg); supplementary doses of 0.1–0.5 g/kg were given when required to maintain a level of anesthesia (average total dose including all additional doses was 1.22 ± 0.05 g/kg, n = 20). We found this dose was adequate in both orexin knockout mice and wild-type mice as judged by baseline stability of AP, HR, respiration, and electroencephalography (EEG) throughout the experiment. Cardiorespiratory parameters were recorded as described in our previous reports (31, 42). In brief, AP was measured by a pressure sensor catheter (SPR-671 microtip pressure catheter for mouse/rat, Millar, Houston, TX) placed in the abdominal aorta through an incision of the right femoral artery. Mean AP (MAP) was calculated by damping 100 Hz–filtered signal. HR was recorded using an HR counter (ET-612J, Nihon Kohden). In both experiments 1 and 2, we did not try to measure EEG because electrical stimulation in the brain makes serious noise in EEG.

In both experiments 1 and 2, the animal was placed in the prone position in a stereotaxic frame (ST-7, Narishige, Tokyo, Japan) so that bregma and lambda would be horizontal. A small hole was drilled on the skull for insertion of a metal electrode or a glass micropipette into the hypothalamus. After completion of surgery, at least 1 h was allowed to stabilize all the parameters. Throughout the experiment, rectal temperature was kept constant at 37.0 ± 1.0°C by a heating pad connected to a thermo controller (ATB-1100, Nihon Kohden).

**Experiment 1: mapping of hypothalamic areas to induce defense response.** We used 18- to 25-wk-old male wild-type mice (n = 10, body wt 32 ± 3 g). Electrical stimulations were
made with stainless steel electrodes. Explored brain regions were the perifornical area and surrounding hypothalamus (from 1.5 to 2.5 mm caudal to the bregma, from 0.3 to 1.5 mm lateral to the midline, and from 4.0 to 5.8 mm ventral to the bregma). An enamel-coated sharp stainless-steel electrode (0.1 mm of the tip exposed) was inserted into the targeted region, and the different lead was placed under the skin of the neck. The electrode was carried in a stereotaxic micromanipulator (SM-11, Narishige) and lowered along the track in steps of 0.3 mm. Three to seven tracks were completed in each animal, and each track was 0.5 mm apart from the other tracks. At each point, the brain was stimulated with a train of rectangular pulses of 0.5-ms duration at 100 Hz for 20 s. The stimulation currents were 0.3 mA with the negative electrode. Stimulations were separated by at least 5 min after all the parameters returned to baseline. We used electrical stimulation in experiment 1 because repeated drug injection to such a tiny brain as in mice cannot be devoid of volume effect (34). However, electrical stimulation excites not only cell bodies but also fibers of passage. Because of such a disadvantage in electrical stimulation, we used the microinjection technique in experiment 2 (see below) to stimulate only cell bodies but not fibers of passage.

**Experiment 2: chemical stimulation of the perifornical area.** Experiments were done in 18- to 25-wk-old male wild-type mice (n = 5, body wt 35 ± 1 g) and prepro-orexin knockout mice (n = 5, 36 ± 2 g). Microinjection of drugs was made with glass micropipettes (Drummond, 1–5 μl) shaped by a micropipette puller (PE-2, Narishige) and cut to fit the tip diameter of 20–30 μm. The pipettes were carried in a stereotaxic micromanipulator (SM-11, Narishige) and connected by silicon tubing to a pressure injector (IM-200J, Narishige). The micropipette was filled with a GABA-A receptor antagonist, bicuculline methiodide (0, 0.1, 0.3, 1.0 mM), dissolved in artificial cerebrospinal fluid (ACSF). The tip of the pipette was positioned in the perifornical area, where maximum response was observed in experiment 1 (2.0 mm caudal to the bregma, 0.65 mm lateral to the midline, 5.0 mm ventral to the bregma, see also Fig. 1). While observing the fluid meniscus in the micropipette through a dissection microscope (OME, Olympus) equipped with an ocular micrometer, a volume of 20 nl was injected by adjusting pressure and time of injection. Bicuculline was administered sequentially from the lowest dose to the highest after recorded parameters returned to the baseline in each animal.

![Fig. 1. Schematic coronal sections of the mouse hypothalamus from 1.5 to 2.5 mm caudal to the bregma showing effects of electrical stimulation on (left to right) consolidated defense response (Total), mean arterial blood pressure (MAP), heart rate (HR), and respiratory frequency (Rf). Data were obtained from 10 wild-type (WT) mice. Arc, arcuate hypothalamic nucleus; DM, dorsomedial hypothalamic nucleus; F, fornix; MT, mammillothalamic tract; VMH, ventromedial hypothalamic nucleus.](image-url)
Experiment 3: measurement of cardiovascular parameters and activity by radiotelemetry. Experiments were done in 30- to 40-wk-old male wild-type mice \( (n = 6, \text{ body wt } 46 \pm 3 \text{ g}) \) and prepro-orexin knockout mice \( (n = 6, 52 \pm 2 \text{ g}) \). A telemetry system (Data Sciences International) was used for measuring AP, HR, and locomotor activity. This system consisted of a radio-frequency transducer (TA11PA-C20) and a receiver. For implantation of the transducer, mice were anesthetized with 2-fluoroethane and given an antibiotic (cephalosporin, 50 mg/kg sc). A midline incision was made on the neck, and the left common carotid artery was isolated. A small cut was made in the vessel wall, and the catheter-transducer was implanted in the thoracic aorta through the hole. The sensor was tunneled subcutaneously and fixed to the abdominal wall. Finally, the incision was closed with sutures. Care was taken to maintain body temperature during and after the surgery. Mice were returned to their home cages and housed individually during the recovery and recording period. All the mice were allowed at least 5 days of recovery from surgery until circadian rhythm was evident in both AP and HR (4).

Given the 24-h period, AP, HR, and activity were recorded for successive 2–3 days using Dataquest LabPRO software (Data Sciences) in unrestrained, freely moving, and unanesthetized conditions.

Two types of stressor were applied in the afternoon on the following day after the basal measurement was completed. The first one was a socioemotional stressor in the resident-intruder test (31). A group-housed, age-matched wild-type mouse (intruder) was placed in the cage for 5 min. When AP, HR, and activity returned to the baseline, a second trial of the resident-intruder test was performed, but the telemeter-implanted animal served as the intruder this time. Cardiovascular and behavioral responses in the first and second trials in the resident-intruder test are called "resident response" and "intruder response," respectively, in this study. The second stressor was noxious stimuli. Vascular clamps of two strengths (60 and 125 g, Fine Science Tools, nos. 18055–01 and -04) were attached to the tail for 10 s.

Finally, on the following day in the light phase, urethane (1.1 g/kg) was intraperitoneally administered to see whether the difference in AP observed in experiment 2 (see RESULTS) could be reproduced and to confirm that attenuation of defense response observed in experiment 2 did not result from generally increased susceptibility of the knockout mice to anesthesia.

Experiment 4: measurement of cardiovascular parameters with indwelling catheter and pharmacological interventions. Experiments were done in 18- to 25-wk-old male wild-type mice \( (n = 21, \text{ body wt } 33 \pm 1 \text{ g}) \) and prepro-orexin knockout mice \( (n = 14, 33 \pm 1 \text{ g}) \). For simultaneous AP measurement and intravascular drug delivery, we used an indwelling catheter to minimize surgical damage because telemetric AP measurement needs additional operation for this purpose. Under isoflurane anesthesia, polyethylene tubing was inserted into the femoral artery for both AP measurement and drug delivery as reported (41). On the following day during the light phase, AP and HR were measured continuously for 2 h under conscious and unrestrained conditions in a quiet environment after at least 1 h of acclimatization. Thereafter, the animals were divided into three groups. The first group was given in a manner that a ganglion blocker, hexamethonium \( (20 \text{ mg/kg, } n = 13 \text{ in wild-type mice and } n = 6 \text{ in knockout mice}) \), to examine possible contribution of the autonomic nervous system in the difference in basal AP between knockout and wild-type mice. The second group was given an angiotensin-converting enzyme inhibitor, captopril (30 mg/kg, \( n = 3 \) in both wild-type and knockout mice), to examine possible interaction of ANG II and orexin, since orexin has been shown to stimulate drinking behavior as potently as ANG II (30). The third group \( (n = 5 \text{ in both wild-type and knockout mice}) \) was sequentially given two drugs: a vasopressin V1a receptor antagonist, \( \beta \)-mercapto-\( \beta \)-cyclpentamethylenepropionyl \( 1 \), O-me-Tyr \( 2 \), Arg \( 3 \)vasopressin \( (10 \mu g/kg) \), and an \( \alpha \)-adrenergic blocker, prazosin \( (1 \text{ mg/kg}) \). Effect of the V1 receptor antagonist was tested because orexin-containing neurons innervate neurons in the paraventricular nucleus containing vasopressin \( (51) \). Resulting AP and HR were calculated as the peak value within 1 h after the administration.

Experiment 5: measurement of echocardiography. Experiments were done in 14- to 18-wk-old male wild-type mice \( (n = 6) \) and prepro-orexin knockout mice \( (n = 6) \). Anesthetized (pentobarbital sodium, 50 mg/kg ip) animals were observed with a 2D-guided M-mode echocardiographic system equipped with a 12-MHz imaging transducer (Sonomos 4500; Agilent Technologies, Osaka, Japan). Left ventricular (LV) diastolic diameter (LVDD) and LV systolic diameter (LVSDD) were measured. LV end-diastolic volume (LVEDV) and LV end-systolic volume (LVESV) were calculated by the cubed method as \( (\text{LVDD}^3 + \text{LVESV}^3)/3 \), respectively. We also calculated percent fractional shortening \( \%\text{FS} = 100 \times (\text{LVDD} - \text{LVESV})/\text{LVDD} \) and ejection fraction \( \%\text{EF} = 100 \times \text{SV}/\text{LVEDV} \). Three beats were averaged for each measurement.

Data analysis and statistical procedure. All the signals were fed into a personal computer (Macintosh, Apple Computer) after analog-to-digital conversion (model 1401, CED, UK or MacLab, AD Instrument, Australia) together with event signals.

In experiment 1, the baseline value was the mean value before stimulation for 20–30 s, and the peak value was calculated as a mean value for 5–10 s around the maximum response.

In experiment 2, data were averaged for every 1 min. The baseline value was the mean value during 5 min before injection of bicuculline. Recovery time was defined as the time from injection until MAP, HR, or RF returned to the value 5% above the baseline, because spontaneous fluctuation of these parameters during control period was within ±5% range of the mean value. We also calculated the area under the curve (AUC) above the baseline value during periods of 10, 15, 20, and 30 min from the injection for 0, 0.1, 0.3, and 1.0 mM of bicuculline, respectively. The length of the periods was determined from the recovery time in wild-type mice (see Table 1). To estimate the cortical arousal level, we calculated the power spectra of EEG using fast Fourier transformation (FTF; length of sections 5 s, FTF number 512). Arousal level was determined as relative \( \beta \)-band (13–50 Hz) power to the total band powers. For relative \( \beta \)-band power, recovery was defined as the time point when the value returned to a value 20% above the baseline, since spontaneous fluctuation of this parameter during control period was within ±20% range of the mean value.

For circadian measurement in experiment 3, AP, HR, and activity counting were averaged for every 30 min. Values in each animal were analyzed by the cosinor method (40) to calculate midline estimating statistic of rhythm (MESOR) and amplitude. The mean light and dark phase values were also calculated. In the resident-intruder test, AP, HR, and activity counting were averaged for every 1 min. AUC was calculated for 5 min when resident and intruder were in the same cage. In the tail-clip test, resulting AP and HR were calculated as the peak value within 30 s after the start of...
clipping. In urethane treatment, pretreatment value was calculated as the mean during the 0.5 h immediately before injection. Posttreatment value was the average during the period from 1 to 6 h after injection, since experiment 2 was usually completed within 6 h.

All data are expressed as means ± SE. Effects of microinjection of bicuculline on cardiovascular, respiratory, and arousal responses were assessed by ANOVA with repeated measures design. Post hoc comparisons of Student-Newman-Keuls procedure or unpaired t-test were used to compare between genotypes. A value of $P < 0.05$ was considered significant.

Histological verification of stimulation sites. At the end of the experiment, the injection site was marked by injecting 20 nl of a 2% Evans blue solution in experiment 2. In experiment 1, the same Evans blue solution was injected to the site where electrical stimulation was finally performed. The animal was deeply anesthetized with additional urethane and transcardially perfused with 20 ml of heparin-added saline followed by 20 ml of 4% formalin. The brains were removed and stored in the formalin solution for at least 2 days before sectioning. Coronal or sagittal sections of 50-μm thickness were cut serially with a micro slicer (DTK-1000, Dosaka EM, Kyoto, Japan), mounted on poly-L-lysine-coated slides, and stained with 1% neutral red or 0.5% cresyl violet. The locations of the injection sites were determined according to the atlas of Paxinos and Franklin (43).

RESULTS

Experiment 1: mapping of hypothalamic areas to induce defense response. To examine whether hypothalamic stimulation induces defense response in mice as in other experimental animals, the effect of electrical stimulation on cardiorespiratory parameters was systematically explored. Ninety-three histologically verified sites within the targeted area were examined in 10 mice. Average MAP, HR, and Rf before stimulation were 115 ± 4 mmHg, 570 ± 15 beats/min, and 194 ± 5/min, respectively. Electrical stimulation in most sites resulted in increases in MAP, HR, and Rf except for 11 sites to which stimulation resulted in decreases in HR. Response magnitude of each parameter was ranked into three levels and is shown in Fig. 1. Stimulation at caudal hypothalamic sites induced a pressor response. The most effective sites were located in the dorsolateral region in the caudal hypothalamus, where electrical stimulation resulted in an increase in MAP by >50 mmHg (Fig. 1). In regard to HR, effective sites were roughly overlapped with but extended more widely compared with the effective sites for MAP. Electrical stimulation at most of the explored sites in the caudal hypothalamus produced an increase in HR by >20 beats/min. An increase in Rf was elicited in the middle to lateral part of the caudal hypothalamus, in which responses in Rf were >300/min.

To map the most effective sites for eliciting defense response, in other words coordinated increases in MAP, HR, and Rf, the rank of response magnitude in each parameter was scored as 2, 1, or 0 points from the highest to the lowest in this order, and summation of scores was calculated for each stimulating site (Fig. 1, left). The total score was highest at three sites in the dorsal part of the perifornical area. The most effective sites were located at 0.65 mm lateral to the midline, 4.7–5.0 mm ventral to the bregma in the plane of 2.0 mm caudal to the bregma, and 1.0 mm lateral, 4.7 mm ventral in the plane of 2.5 mm caudal to the bregma.

These results clearly show that defense response can be elicited by electrical stimulation of dorsal hypothalamus, especially the dorsal part of the perifornical area in mice. From these results, we decided to stimulate at a point 2.0 mm caudal to the bregma, 0.65 mm lateral to the midline, and 5.0 mm ventral to the bregma in the following experiment.

Experiment 2: chemical stimulation of the perifornical area in orexin knockout mice. To test our hypothesis that orexin may contribute to expression of the defense response, we compared the effects of chemical stimulation with bicuculline to the perifornix on cardiorespiratory and EEG parameters between orexin null-mutated knockout mice and wild-type littermates. Before stimulation, the average values of MAP, HR, Rf, and relative β-power in five wild-type mice were 117 ± 8 mmHg, 558 ± 12 beats/min, 178 ± 7/min, and 14 ± 2%, respectively. In wild-type mice, microinjection of 0.1–1 mM of bicuculline to the perifornical area elicited dose-dependent increases in MAP, HR, Rf, and relative β-power (Fig. 2), but ACSF (vehicle) did not cause any significant changes. By 0.1, 0.3, and 1.0 mM of bicuculline, peak increases in MAP (11 ± 2%, 13 ± 1, and 28 ± 2%), HR (13 ± 3, 17 ± 2, and 19 ± 4%), Rf (54 ± 6, 103 ± 7, and 167 ± 15%), and relative β-power (111 ± 22, 122 ± 39, and 121 ± 17%) were all statistically significant. These changes began within 15 s from injection of bicuculline and returned to baseline within 10–30 min depending on the dosage (Table 1).

In orexin knockout mice, basal values of MAP, HR, Rf, and relative β-power were 92 ± 3 mmHg, 562 ± 22 beats/min, 218 ± 11/min, and 13 ± 3%, respectively. MAP was significantly ($P < 0.05$) lower and Rf was significantly higher than that in wild-type mice (Fig. 2). Basal HR and relative β-power were not significantly different between the genotypes. At a glance, microinjection of bicuculline to the perifornical area in the mutant mice appeared to result in similar cardio-

Table 1. Duration of responses by bicuculline

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Dose of Bicuculline, mM in 20 nl</th>
<th>Wild-Type Mice</th>
<th>Knockout Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood pressure</td>
<td>0.1</td>
<td>10.8 ± 2.6</td>
<td>2.6 ± 1.7*</td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>15.8 ± 2.9</td>
<td>5.0 ± 1.6*</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>26.0 ± 2.7</td>
<td>14.0 ± 2.2*</td>
</tr>
<tr>
<td>Heart rate</td>
<td>0.1</td>
<td>12.4 ± 1.6</td>
<td>1.8 ± 1.1*</td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>22.8 ± 2.4</td>
<td>9.0 ± 2.5*</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>31.8 ± 2.3</td>
<td>25.2 ± 5.5</td>
</tr>
<tr>
<td>Respiratory frequency</td>
<td>0.1</td>
<td>12.4 ± 1.6</td>
<td>1.6 ± 1.0*</td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>19.2 ± 2.8</td>
<td>9.4 ± 1.4*</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>21.2 ± 2.6</td>
<td>19.6 ± 5.7</td>
</tr>
<tr>
<td>EEG β-band power</td>
<td>0.1</td>
<td>11.0 ± 1.0</td>
<td>2.2 ± 0.9*</td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>18.8 ± 2.2</td>
<td>8.6 ± 2.6*</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>28.2 ± 4.7</td>
<td>11.0 ± 1.4*</td>
</tr>
</tbody>
</table>

Values are means ± SE of response duration in min. *$P < 0.05$ vs. wild-type mice (repeated-measure ANOVA followed by post hoc test of Student-Newman-Keuls).

The most effective sites were located in the dorsolateral region in the caudal hypothalamus, where electrical stimulation resulted in an increase in MAP by >50 mmHg (Fig. 1). In regard to HR, effective sites were roughly overlapped with but extended more widely compared with the effective sites for MAP. Electrical stimulation at most of the explored sites in the caudal hypothalamus produced an increase in HR by >20 beats/min. An increase in Rf was elicited in the middle to lateral part of the caudal hypothalamus, in which responses in Rf were >300/min.

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In orexin knockout mice, basal values of MAP, HR, Rf, and relative β-power were 92 ± 3 mmHg, 562 ± 22 beats/min, 218 ± 11/min, and 13 ± 3%, respectively. MAP was significantly ($P < 0.05$) lower and Rf was significantly higher than that in wild-type mice (Fig. 2). Basal HR and relative β-power were not significantly different between the genotypes. At a glance, microinjection of bicuculline to the perifornical area in the mutant mice appeared to result in similar cardio-

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respiratory responses to those in wild-type mice (Fig. 2). In detailed analysis, however, there was a significant quantitative difference between knockout mice and wild-type mice. Whereas 0.1 mM of bicuculline was effective to elicit significant changes in MAP, HR, and Rf in wild-type mice, the same dose of bicuculline did not cause any significant changes in the knockout mice (Fig. 2, A–D, left), except for a small and shortlasting increase in HR. At the dose of 0.3 mM, cardiorespiratory responses in the knockout mice were shorter lasting than those in wild-type mice (Fig. 2, A–D, middle, and Table 1). At the dose of 1.0 mM, responses in AP and EEG were still shorter lasting although recovery time of HR and Rf responses was not statistically different from the wild-type mice (Fig. 2, A–D, right, and Table 1). Consequently, response magnitudes as calculated by AUC were significantly smaller in knockout mice for all the parameters (Fig. 2E) at least for 0.1 and 0.3 mM of bicuculline.

By histological examination, there was no difference in the dye distribution between wild-type mice and orexin knockout mice, and all the injections were successfully made in dorsal part of the perifornical area (Fig. 3).

Experiment 3: measurement of cardiovascular parameters and activity by radiotelemetry. Attenuation of defense response in orexin knockout mice was further confirmed by radiotelemetric measurement of behavioral and cardiovascular parameters in unanesthetized freely moving mice with a natural stressor (Fig. 4). At the baseline before stress, low AP and similar HR in orexin knockout mice were observed as was the case in anesthetized condition. When the test animal was confronted with an intruder, increases in AP, HR, and activity were significantly smaller in orexin knockout mice than in wild-type mice (Fig. 4A). The same was true when the test animal intruded into a resident’s home cage (Fig. 4B). On the other hand, noxious stimuli of the tail pinch elicited similar increases in AP and HR in both genotypes, irrespective of stimulus intensity (Fig. 5).

We next examined circadian rhythm of AP, HR, and activity in the orexin knockout mice, because deficiency of orexin has been shown to induce disturbance in sleep-awake cycle and thus may induce abnormality in circadian rhythm of cardiovascular parameters. Activity during dark phase but not during light phase was significantly lower in orexin knockout mice and thus amplitude of 24-h fluctuation in activity was significantly lower in the mutant mice (Fig. 6, Table 2). Contrary to our expectation, however, circadian fluctuation of AP and HR was similar between knockout and wild-type mice except that AP in knockout mice was consistently lower during both dark phase and light phase. Although amplitude of AP in orexin knockout mice tended to be smaller, the difference did not reach statistical significance ($P = 0.34$).

To exclude the possibility that basal hypotension in orexin knockout mice in experiment 2 was due to a difference in sensitivity to anesthetic, we next examined the effect of urethane administration on AP and HR in radiotelemeber-indwelling mice. As expected, urethane lowered AP in a similar manner in mutant (before urethane, 108 ± 3 mmHg and after, 83 ± 5 mmHg, −23.5 ± 3.5%) and wild-type mice (before urethane, 127 ± 6 mmHg and after, 102 ± 7 mmHg, −19.7 ± 3.6%). HR was not different between knockout
mice and wild-type mice either before or after injection of urethane (data not shown).

**Experiment 4: measurement of cardiovascular parameters with indwelling catheter and pharmacological interventions.** Lower basal AP in orexin knockout mice was again confirmed in this experiment (Fig. 7A). Basal HR of both genotypes (Fig. 7B) was higher than that in experiment 3 where cardiovascular parameters were measured with radiotelemeter (Table 2). Nevertheless, there was no difference in HR between the knockout mice and wild-type mice as was the case in experiment 3.

To examine why basal AP was lower in orexin knockout mice, possible abnormalities of the renin-angiotensin system, vasoconstriction through vasopressin V1 receptor, or autonomic nervous system were evaluated using captopril, V1 antagonist, hexamethonium, and prazosin. Administration of captopril lowered AP in both mutant (\(-12.0 \pm 1.7\%, n = 3\)) and wild-type mice (\(-11.9 \pm 2.7\%, n = 3\)) in a similar manner (\(P > 0.05\)). Resultant AP was still lower in knockout mice than in wild-type mice, although the difference did not reach statistical significance presumably because of small numbers of the animals. V1 antagonist did not affect AP in either mutant (\(-4.6 \pm 2.5\%, n = 5\)) or wild-type mice (\(-2.6 \pm 1.1\%, n = 5\)). Thus resultant AP was still significantly lower in knockout mice (Fig. 7A). On the other hand, hexamethonium lowered AP more in wild-type (\(-32.1 \pm 2.1\%, n = 13\)) than in mutant mice (\(-23.4 \pm 2.6\%, n = 6; P < 0.05\)). Resulting AP was not different between the two. In a similar manner, prazosin lowered AP greater in wild-type (\(-62.2 \pm 3.1\%, n = 5\)) than in mutant mice (\(-51.2 \pm 2.5\%, n = 5; P < 0.05\)). Difference in AP was canceled after the treatment with prazosin (Fig. 7A). There was no difference in HR between the two strains with any drugs used in the current experiment (Fig. 7B).

**Experiment 5: measurement of echocardiography.** To examine the possibility that cardiac abnormality contributed to the lower AP in orexin knockout mice, we measured echocardiography in another set of the animals. Stroke volume and other basal contractile parameters were not significantly different between the two groups (Table 3).

**DISCUSSION**

We demonstrated here that electrical stimulation and disinhibition of GABAergic input to the perifornical area elicited the cardiovascular and respiratory defense responses and increased arousal level in urethane-anesthetized mice in a similar manner as in cats (1), rabbits (49), and rats (13, 57). A recent report that spontaneous activity of identified orexin-containing neuron in slice preparation was inhibited by the GABA-A receptor agonist muscimol supports the relev-
enous orexins play a role in cardiovascular and respiratory regulations in the central nervous system.

Present results confirmed the proposal of possible contribution of orexin in cardiovascular regulation (3, 7, 15, 26, 36, 47, 50) and in stress-induced behavior (27, 55). In other words, our present results using knockout mice were generally in accordance with the earlier reports using exogenous application of orexin and determination of orexin content in the brain. Moreover, we successfully linked cardiovascular and stress regulatory roles of orexin by showing attenuation of defense response in orexin knockout mice in both anesthetized and freely moving conditions.

We demonstrated here diminished defense response in orexin knockout mice, but the response was not completely abolished. Preserved responses, namely short-lasting cardiorespiratory excitation at low doses of bicuculline and even similar responses in HR and Rf at high dose of bicuculline (Fig. 2), should be mediated by other transmitter(s)/modulator(s) than orexin. Existence of other factors than orexin is further suggested by incomplete disappearance of cardiovascular and behavioral responses in resident-intruder test (Fig. 4).

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Table 2. Circadian statistics and values for light and dark phases

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wild-Type Mice</th>
<th>Knockout Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood pressure, mmHg</td>
<td>125 ± 6</td>
<td>109 ± 2*</td>
</tr>
<tr>
<td>MESOR</td>
<td>9 ± 2</td>
<td>6 ± 1</td>
</tr>
<tr>
<td>Amplitude</td>
<td>0.03 ± 0.56</td>
<td>1.20 ± 0.46</td>
</tr>
<tr>
<td>Acrophase (h: min)</td>
<td>122 ± 6</td>
<td>107 ± 2*</td>
</tr>
<tr>
<td>Light phase mean</td>
<td>128 ± 6</td>
<td>113 ± 2*</td>
</tr>
<tr>
<td>Heart rate, min⁻¹</td>
<td>529 ± 41</td>
<td>565 ± 22</td>
</tr>
<tr>
<td>MESOR</td>
<td>35 ± 8</td>
<td>34 ± 4</td>
</tr>
<tr>
<td>Acrophase (h: min)</td>
<td>0.48 ± 0.42</td>
<td>2.50 ± 0.49</td>
</tr>
<tr>
<td>Light phase mean</td>
<td>527 ± 28</td>
<td>553 ± 23</td>
</tr>
<tr>
<td>Dark phase mean</td>
<td>554 ± 38</td>
<td>575 ± 21</td>
</tr>
<tr>
<td>Activity, counts/30 min</td>
<td>135 ± 24</td>
<td>84 ± 17</td>
</tr>
<tr>
<td>MESOR</td>
<td>79 ± 20</td>
<td>25 ± 4*</td>
</tr>
<tr>
<td>Acrophase (h: min)</td>
<td>2.09 ± 0.43</td>
<td>2.48 ± 0.13</td>
</tr>
<tr>
<td>Light phase mean</td>
<td>98 ± 17</td>
<td>78 ± 17</td>
</tr>
<tr>
<td>Dark phase mean</td>
<td>162 ± 26</td>
<td>91 ± 17*</td>
</tr>
</tbody>
</table>

Values are means ± SE of 6 mice in each genotype. MESOR, midline estimating statistic of rhythm. *P < 0.05 vs. wild-type mice (t-test).

Perhaps glutamate may be one of such transmitters, since bilateral microinjection into the RVLM of an ionotropic excitatory amino acid receptor antagonist kynurenic acid attenuated an increases in AP and renal sympathetic nerve activity evoked by air jet stress (37) or by direct hypothalamic stimulation (52).

Table 3. Echocardiography parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wild-Type Mice</th>
<th>Knockout Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>30.3 ± 0.7</td>
<td>29.0 ± 1.1</td>
</tr>
<tr>
<td>LV diastolic diameter, mm</td>
<td>3.54 ± 0.06</td>
<td>3.45 ± 0.13</td>
</tr>
<tr>
<td>LV systolic diameter, mm</td>
<td>1.99 ± 0.06</td>
<td>1.99 ± 0.08</td>
</tr>
<tr>
<td>LV end-diastolic volume, μl</td>
<td>46.6 ± 2.2</td>
<td>43.7 ± 4.9</td>
</tr>
<tr>
<td>LV end-systolic volume, μl</td>
<td>8.29 ± 0.69</td>
<td>8.42 ± 1.14</td>
</tr>
<tr>
<td>Stroke volume, μl</td>
<td>38.3 ± 1.7</td>
<td>35.3 ± 3.9</td>
</tr>
<tr>
<td>Fractional shortening, %</td>
<td>43.9 ± 1.1</td>
<td>42.4 ± 0.8</td>
</tr>
<tr>
<td>Ejection fraction, %</td>
<td>82.3 ± 1.0</td>
<td>80.8 ± 0.8</td>
</tr>
</tbody>
</table>

Values are means ± SE of 6 mice in each genotype. No parameters were significantly different between orexin-knockout and wild-type mice. LV, left ventricular.

Orexin was able to increase pre- and postsynaptic activity of the glutamate-releasing neurons (54). Thus orexin may act as a modulator of glutamatergic input into the RVLM. A toxin-induced orexin-neuron specific degenerative animal model (21) may help to study other factors, especially cotransmitters contained in the orexin neuron, involved in defense response. On the other hand, above-cited reports dealt with only the cardiovascular components of the defense response. There is little information about neurotransmitter(s) that convey respiratory or behavioral component of the defense response. In this respect, this is the first report suggesting a possible neurotransmitter/modulator that contributes to the simultaneous and coordinated changes in cardiovascular, respiratory, EEG, and behavioral components of the defense response.

The present study did not clarify the site at which orexin acted in the central nervous system and the mechanisms of cardiovascular and respiratory responses induced by disinhibition of GABAergic neurotransmission in the perifornical area. However, previous studies enabled us to suppose key sites where orexin acted as a mediator of defense response. The most apparent site seems to be the RVLM, since inhibition of RVLM or subjacent ventral surface of the medulla (glycine-sensitive area) attenuated sympathetic and defense responses evoked by stimulation to the lateral hypothalamic area (12, 24, 52). A double-virus transneuronal labeling technique revealed that not only perifornical neurons but also RVLM neurons provided a dual input to the sympathetic outflow systems that regulate cardiac and adrenal medullary functions, indicating these neurons were the “central command neurons” of defense response (28). Actually, orexin immunoreactive fibers were seen in the RVLM (44), and exogenous applied orexin into the RVLM elicited AP and sympathetic excitation (7, 36). Present results with hexamethonium and prazosin also support the view since RVLM is the major source of sympathetic outflow (8). Nevertheless, we cannot exclude the possible involvement of sites other than RVLM, such as periaqueductal gray, nucleus tractus solitarius, or intermediolateral cell column of the spinal cord (2, 26), as active sites of orexin in the defense response. This subject needs further experimentation.

In clear contrast to attenuated cardiovascular responses to socioemotional stress by resident-intruder...
test, AP and HR responses to noxious stimuli by tail pinch were not different between orexin knockout mice and wild-type mice (Fig. 5). This may be because stimulus duration was shorter in tail pinch (10 s) than in resident-intruder test (5 min). Another explanation may be that cardiovascular responses to noxious stimuli by tail pinch do not always require supraspinal structures (48) and hence the responses are independent from hypothalamic orexin. On the other hand, intrathecal administration of orexin induced analgesia in rats (56). Analgesia but not exaggerated pain response may be advantageous to defense response. Thus orexin may also participate in stress-induced analgesia. Although this hypothesis should be clarified in future experiments, we can say, at present, that the orexin system may be activated by some but not all kinds of stressors. We cannot exclude the possibility that absence of orexin resulted in some deficit in sensory system for socioemotional stress but not for noxious stressor. Nevertheless, results from urethane-anesthetized mice with direct hypothalamic stimulation point to a probable deactivation of AP through tonic excitation of sympathetic efferent pathway.

Circadian rhythm of AP and HR, calculated as phase and amplitude, was not significantly different between orexin knockout mice and wild-type mice although activity during dark phase was less in the mutant mice (Fig. 6, Table 2). The latter observation was consistent with previous studies showing reduced awake time and increased sleep time during dark phase in orexin neuron-ablated mice (21). It is not clear from the present experiment why amplitudes of circadian fluctuations of AP and HR were not different between the two strains while that of activity was smaller in orexin knockout mice. Activity may not be the sole determinant of AP and HR. Whatever may be the reason, an important point is that AP in orexin knockout mice was smaller than wild-type mice even during the light phase when both animals were at rest and activities were not different between the two. The difference in AP was also independent of HR or cardiac contractility (Table 3). The cause of the difference in AP seemed to be attenuated sympathetic outflow that regulates peripheral vascular resistance in orexin knockout mice (Fig. 7). Basal cardiac sympathetic nerve activity seems not influenced by the absence of orexin. Contribution of the renin-angiotensin system and vasopressin V1 receptor seemed minimal, although small numbers of the animals prevented us from making conclusive remarks about the former. Our conclusion is in line with sympathetic excitation by exogenously administered orexin (3, 15, 50) and extended possible contribution of orexin to basal determination of AP through tonic excitation of sympathetic nervous system.

**Perspectives**

The difference in AP between two genotypes was maintained after urethane anesthesia and attenuated defense response was observed in both awake and anesthetized conditions. This suggests that the difference in basal AP cannot be explained by possible attenuation of defense response in orexin knockout mice because defense response is not always activated in daily life or under anesthesia. It is possible that there may be two subgroups of orexin-containing neurons: one group contributes to determination of basal AP and another participates in defense response, since orexin-containing neurons widely distribute in lateral hypothalamus and dorsomedial hypothalamus (39) and subpopulations of orexin neurons have been proposed in respect to responses to psychotic drugs (18). However, orexin neurons may be activated only by arousal without any particular stress (17). Therefore, it is also possible that the same orexin system contributes to both basal AP determination by mild activation and to defense response when further activated by stress. We do not have the answer at present on this issue.

There are only a few reports describing autonomic regulation in narcolepsy patients. Sachs and Kajisera (45) reported that never-medicated narcoleptic patients showed attenuated autonomic reflexes (changes in AP and HR) in handgrip test and Valsalva’s maneuver, but not in face immersion test or orthostatic standing. Because some but not all reflexes had been disturbed, they proposed intact peripheral nerves and a localization of the defect to the central nervous system. Our findings of attenuated defense response and preserved pain-induced response in orexin-deficient mice are in accordance with their findings in human narcolepsy. Basal AP in narcolepsy patients is rather controversial. The same authors reported normal AP and HR at rest before the autonomic testing (45). However, Guilleminault (20) reported that withdrawal of medication with amphetamine for 4 wk significantly decreased AP in narcoleptic patients, indicating low AP, otherwise taking a central stimulant (20). To the best of our knowledge, 24-h AP and HR have not been reported in narcoleptic patients. We feel that systematic reinvestigation about autonomic regulation in narcoleptic patients is needed, since these reports cited here appeared before 1999 when deficiency of orexin had been revealed as the cause of narcolepsy.

In summary, we found that defense response could be elicited in mice as in the other experimental animals by stimulation to the perifornical region of the posterior hypothalamus. Anesthetized prepro-orexin knockout mice showed lower blood pressure and faster respiratory frequency than those in the wild-type mice and attenuated defense response evoked by microinjection of bicuculline. Attenuated defense response and hypotension was also reproduced in unanesthetized freely behaving mutant mice. The hypotension observed might be due to an attenuated sympathetic outflow. The present study suggests that orexin-containing neurons in the perifornical area play a role for one of the efferent pathways of defense response. Moreover, intrinsic orexin contributes to the maintenance of basal blood pressure.
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DISCLOSURES

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