Reduced brown adipose tissue thermogenesis during environmental interactions in transgenic rats with ataxin-3-mediated ablation of hypothalamic orexin neurons

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1Centre for Neuroscience, Department of Human Physiology, Flinders University, Adelaide, South Australia, Australia; and 2International Institute for Integrative Sleep Medicine (WPI-IIIS), University of Tsukuba, Japan; and Department of Molecular Genetics, University of Texas Southwestern Medical Center, Dallas, Texas

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Mohammed M, Ootsuka Y, Yanagisawa M, Blessing W. Reduced brown adipose tissue thermogenesis during environmental interactions in transgenic rats with ataxin-3-mediated ablation of hypothalamic orexin neurons. Am J Physiol Regul Integr Comp Physiol 307: R978–R989, 2014.—Thermogenesis in brown adipose tissue (BAT) contributes to substantial increases in body temperature evoked by threatening or emotional stimuli. BAT thermogenesis also contributes to increases in body temperature that occur during active phases of the basic rest-activity cycle (BRAC), as part of normal daily life. Hypothalamic orexin-synthesizing neurons influence many physiological and behavioral variables, including BAT and body temperature. In conscious unrestrained animals maintained for 3 days in a quiet environment (24–26°C) with ad libitum food and water, we compared temperatures in transgenic rats with ablation of orexin neurons induced by expression of ataxin-3 (Orx_Ab) with wild-type (WT) rats. Both baseline BAT temperature and baseline body temperature, measured at the onset of BRAC episodes, were similar in Orx_Ab and WT rats. The time interval between BRAC episodes was also similar in the two groups. However, the initial slopes and amplitudes of BRAC-related increases in BAT and body temperature were reduced in Orx_Ab rats. Similarly, the initial slopes and amplitudes of the increases in BAT temperatures induced by sudden exposure to an intruder rat (freely moving or confined to a small cage) or by sudden exposure to live cockroaches were reduced in resident Orx_Ab rats. Constriction of the tail artery induced by salient alerting stimuli was also reduced in Orx_Ab rats. Our results suggest that orexin-synthesizing neurons contribute to the intensity with which rats interact with the external environment, both when the interaction is “spontaneous” and when the interaction is provoked by threatening or salient environmental events.

emotional hyperthermia; body temperature; basic rest-activity cycle; cutaneous blood flow; thermoregulation; stress; ultradian rhythm

HYPOTHALAMIC OREXIN (hypocretin)-synthesizing neurons (14, 49) influence physiological and behavioural processes associated with daily life, including arousal and changes in body temperature (3, 11, 13, 17, 18, 20, 26, 32, 33, 46, 60, 66, 67). The role of orexin-synthesizing neurons has been elucidated by experiments using transgenic mice with ataxin-3-mediated destruction of orexin-synthesizing neurons (18). These animals have attenuated thermogenic responses to handling stress (66) and to exposure to cold (57).

The daily life of Sprague-Dawley rats is organized into active and inactive phases. Kleitman (24) described this organization of physiological and behavioral events as the basic rest-activity cycle (BRAC). Our laboratory has shown that centrally programmed increases in brown adipose tissue (BAT) thermogenesis contribute to BRAC-associated changes in temperature (44). BAT thermogenesis commences at the onset of an active phase of the BRAC, and eating usually commences ~15 min after the onset of BAT thermogenesis (5, 6).

BAT thermogenesis also contributes to the rise in body temperature initiated by threatening environmental occurrences (emotional hyperthermia) (29, 35). The occurrence of potentially threatening or salient environmental events also acutely decreases thermoregulatory cutaneous blood flow (34, 35, 63, 64). We refer to these sudden changes in thermoregulatory cutaneous blood flow as sympathetic cutaneous vasoconstricitive responses (SCVARs) (15).

The availability of transgenic rats with ataxin-3-mediated destruction of orexin-synthesizing neurons (3) has provided an opportunity for studies of the physiological and behavioral roles of orexin-synthesizing neurons during daily activities in this species. To date, experiments with these animals have concentrated on sleep physiology, with information on body temperature limited to the effects of sleep/wake states on this parameter (3, 51, 65). So far there are no reports of the responses of these transgenic rats to emotional stresses, nor are there reports of BRAC in these animals.

The present study compared BAT and body temperature as well as tail artery blood flow in conscious freely moving transgenic rats with ataxin-3-mediated destruction of orexin-synthesizing neurons (Orx_Ab rats) and in their wild-type (WT) littermates in different experimental models involving interactions with the external environment. The BRAC organization of daily life, including changes in BAT, body temperature, and food intake, was examined in animals left undisturbed with ad libitum food and water in a 24–26°C environment with a 12:12-h dark-light cycle with ad libitum access to food and water for 4 days. We suddenly introduced a Sprague-Dawley intruder rat, either freely moving or confined to a small cage, into the cage of the resident Orx_Ab or WT rat. We also suddenly introduced mesh-enclosed live cockroaches into the cage of Orx_Ab or WT rats (39). Finally, we compared tail artery vasoconstriction elicited by salient alerting events (SCVARs) in Orx_Ab and WT rats. We hypothesized that BAT thermogenesis and tail artery vasoconstriction normally initiated during interactions with the external environment would be reduced in Orx_Ab rats. To validate our use of BAT temperature as an index of BAT thermogenesis, we also compared BAT and body temperature parameters in WT rats.
during BRAC-related or intruder-induced increases in temperature. We hypothesized that the slope of the initial increase in BAT temperature would be greater than the slope of the increase in body temperature and that the amplitude of the increase in BAT temperature would be greater than the corresponding value for body temperature.

MATERIALS AND METHODS

Genotyping and Immunohistochemical Verification of Phenotype

Orx_Ab transgenic Sprague-Dawley rats were initially received from M. Yanagisawa’s laboratory at Howard Hughes Medical Institute (Dallas, TX). The generation of these transgenic rat lines has been previously reported (3). Orx_Ab rats have the ataxin-3 gene attached to the downstream end of the human preproorexin promoter gene, which was then inserted into pronuclei of fertilized Wistar rat eggs to generate founder animals (Orx_Ab). These animals were back crossed into Sprague-Dawley rats.

At Flinders University, we established a breeding colony from heterozygous Orx_Ab rats (ataxin-3/−) paired with WT rats (−/−). The colony was maintained by back crossing with normal Sprague-Dawley rats. In all rats used in our experiments, DNA samples were extracted from the ear notches, and the genotypes were confirmed by PCR. The primers used against the human preproorexin promoter region were 5′-GCAGCGGCCATTCCTTGG-3′ and 5′-CAGCGTAAATCTGGGAACATCGTATGGG-3′, and the primers used against rat preproorexin were 5′-GCACCGAAGATACCATCTCTC-3′ and 5′-GACCTGGATCCGCCCCGGGCCTA-AAGC-3′.

Immunohistochemistry

After completion of the experiments (see below), rats were anesthetized with pentobarbital (100 mg/kg ip), and brains were perfused transcardially with aldehyde fixatives, removed, and left in the fixative with 30% sucrose. Serial sections (50 μm) were cut from the forebrain using a freezing microtome (Leitz) and washed thoroughly with 50% ethanol. Tissues were then incubated overnight at 4°C in rabbit anti-orexin antisera (1:5,000, Peptide Institute, Minohshi, Japan). After incubation in primary antibody, sections were washed with 1% normal horse serum-Tris-buffered saline and then incubated in biotinylated goat anti-rabbit IgG antibody (1:200, Vector Laboratories) overnight at 4°C. Sections were treated with avidin-biotin complex (Vectastain ABC kit, Vector Laboratories, Burlingon, CA) for 1 h, reacted with 0.05% diaminobenzidine for 10 min, and then incubated with 0.01% H2O2. Tissues sections were then mounted on gelatin-coated microscopic slides, dehydrated with graded alcohol, and covered slipped for histological examinations in both light and dark fields.

Animals and Surgical Procedures

Experiments approved by the Animal Welfare Ethical Committee of Flinders University were carried out on 4- to 5-mo-old WT (n = 23, mean weight: 434 ± 14 g) and Orx_Ab (n = 24, mean weight: 423 ± 12 g, P > 0.05 vs. the weight of WT rats) male Sprague-Dawley rats. Care was taken to minimize the number of animals used. For implantation of measuring devices, rats were anesthetized with 2% isoflurane (Veterinary Companies of Australia) in 100% O2. Thermistors were chronically implanted in the interscapular BAT and in the mediastinum just ventral to the trachea to continuously record BAT and body temperatures (6). Thermistor cables were passed subcutaneously and attached to a head socket screwed to the skull. An ultrasonic Doppler flow probe (Iowa Doppler Products) was implanted around the tail artery ~2 cm distal to the base (16, 44). Flow probes were connected via subcutaneous wires to the same headpiece attached to the skull. Rats were left undisturbed for 1 wk to recover from surgical stress.

Experimental Protocols

BRAC experiments. Orx_Ab and WT rats were transferred into sound-insulated, temperature-controlled (24–26°C) experimental cages, with lights on at 1900 hours and off at 0700 hours and food and water available ad libitum. BAT and body temperatures and tail blood flow signals were acquired from the head socket via a swivel device. Gross behavioral activity was measured using infrared grids (25). The food container was suspended from a strain gauge with a frequency response so that both timing and amount of meals could be measured (6). Rats were left undisturbed for 4 days. Records for the last 3 days were used in our analysis.

Sudden confrontation with an intruder rat. Instrumented WT and Orx_Ab rats were individually housed as for the BRAC experiments (described above). BAT and body temperatures were continuously recorded for at least 12 h. When a stable baseline was observed, a second rat (an un instrumented male Sprague-Dawley rat, weight: 350–450 g) was introduced into the experimental cage for a period of 30 min. In one group of animals, the intruder rat was able to move freely inside the resident rat’s cage. In another group, the intruder rat was confined to a smaller cage, preventing physical contact with the resident rat (35).

Introduction of cockroaches inside the experimental cage. Orx_Ab and WT rats were left undisturbed overnight without food pellets with free access to water. The next day, the cage of the resident rat was suddenly opened, and a mesh sack containing four to five live cockroaches (Periplaneta australasiae) was hooked to the outside of the empty food container to provide the rat with a salient stimulus (39). The cage was then closed, and BAT and body temperatures and tail blood flows were monitored for 30 min. The sack of cockroaches was then removed.

Alerting stimuli and tail artery constrictions. Orx_Ab and WT rats were transferred into a wooden cage (40 × 40 × 40 cm), temperature: 24–26°C fitted with a swivel device. The tail artery Doppler blood flow signal was continuously recorded for 30 min. Standardized alerting stimuli (see below) were then administered at times when the tail artery blood flow was at a high level. The following alerting stimuli were delivered in a constant order with at least 5-min intervals: 1) a flexible metal rod was released from a restraint so that it suddenly tapped the side of the cage, 2) the cage was dropped by 1.5 cm, 3) the box was vigorously moved to and fro two to three times, and 4) a small window (15 × 15 cm) in the front of the cage was suddenly opened.

Data Recordings and Statistical Analysis

Animals were connected to the swivel device with a flexible cable and a counterbalanced swivel device (SL12C, PlasticsOne, Roanoke, VA) at the top of the cage. Temperature signals (BAT and body temperatures) were passed to a bridge amplifier (BME, Flinders University) and then digitized (1 Hz) with PowerLab (AD Instruments, Castle Hill, NSW, Australia). The tail artery Doppler signal was transmitted to the head piece and then, via the flexible cable and swivel device, to a System 6 model 200 device (Triton Technology, San Diego, CA), which converted the frequency difference to voltage. The voltage signal was calibrated (in cm/s) using the Triton internal calibrator. The voltage signal was then transferred to a PowerLab device programmed with Chart software (AD Instruments) for signal sampling (40 Hz) and analog-to-digital conversion. Chart data were then exported to IgorPro software (Wavemetrics).

In BRAC experiments, IgorPro software was used to identify individual BRAC episodes using the procedure described in our previous studies (5, 6, 44). Onset times and values as well as amplitudes and durations of BAT and body temperature BRAC episodes were analyzed as described in our previous papers (6, 44). We calculated BAT and body temperature maximum slopes during BRAC episodes in the following manner. We first averaged, for each rat, all the initial 5-min epochs of each BRAC episode for all 3 days,

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separately for dark and light periods. For each rat, we then calculated the maximum slope of this averaged signal. In each experimental condition, slope values were then averaged across rats. Behavioral activity from the onset of the each BRAC episode to the onset of eating was measured from the infrared grid (6). Time and amount of food eaten were analyzed, as previously reported (6). We also used the continuous wavelet transform (CWT) function in IgorPro to analyze BAT and body temperature records for light and dark periods of the third observation day. The CWT function uses a modification of the Fourier transform to provide an index of the spectral content of the records at different frequencies of occurrence of BRAC episodes (27, 28, 47).

For intruder rat and cockroach experiments, BAT and body temperatures data were obtained as 1-Hz signals, commencing 10 min before the introduction of the intruder rat or cockroaches and finishing 30 min later. We used IgorPro to calculate the average slope of the linear fit to BAT and body temperature signals for individual resident rats for the initial 5 min after the introduction of the intruder rat or cockroaches. Amplitudes of the increases in BAT and body temperature were obtained by subtracting the immediate preintroduction temperature from the temperature recorded between 26 and 30 min after the introduction. In the cockroach experiments, we also recorded tail artery blood flow.

For SCVAR experiments, we quantified the change in the tail artery Doppler flow signal using our previously described SCVAR index (15, 34). We selected 3 s of the tail blood flow signal just before the administration of individual alerting stimuli and 3 s at the lowest flow level in the 10 s after the stimulus presentation. The SCVAR index uses both mean blood flow and average amplitude of each individual tail artery pulse (0.25-s bins) to calculate an index of the percent fall in blood flow induced by the alerting stimulus. The SCVAR index formula is 100 - [(poststimulus mean flow + poststimulus mean pulse amplitude)/(prestimulus mean flow + prestimulus mean pulse amplitude)] × 100, so that a large fall in the Doppler flow signal leads to a high SCVAR index, indicating a large percent fall in flow.

Group data are presented as means ± SE. Group results were analyzed statistically using Statview 2 software (SAS Institute, Carey, NC). In both Orx_Ab and WT rats, mean temperature and tail blood flow parameters as well as activity and food intake were compared using factorial ANOVA. Repeated-measures ANOVA was used to analyze differences between BAT and body temperatures recorded simultaneously in the same rat.
similar (Fig. 3). This similarity in the pattern of onset intervals is also shown in Fig. 4.

There were no differences between Orx_Ab and WT animals in BAT or body temperatures measured at the onset of BRAC episodes (baseline temperatures) in either dark or light periods (Table 1). The maximum slope of the initial 5 min of the BAT temperature records during a BRAC episode in Orx_Ab rats was less than the corresponding maximum slope in WT rats (Table 1 and Fig. 5) for both dark and light periods. The amplitude of the BRAC-associated increases in BAT and body temperature were less in Orx_Ab rats compared with WT rats for both dark and light periods (Table 1). The durations of BRAC-associated increases in BAT temperature were less in Orx_Ab rats compared with WT rats during the dark period, but for the light period, the durations were similar (Table 1).

The overall CWT power of the BAT temperature traces was reduced in Orx_Ab rats compared with WT rats for both dark and light periods (Table 1). The power of the body temperature traces was reduced in Orx_Ab rats for the dark period, but the values for the light period were similar in Orx_Ab and WT rats (Fig. 4 and Table 1).

**Relation Between BAT Temperature and Food Intake During BRAC**

Behavioral activity during the period between the onset of BAT thermogenesis and commencement of eating, the active phase of BRAC, was reduced in Orx_Ab rats (Table 2). Orx_Ab rats ate less than WT rats (Table 2). The ~15-min time delay between the onset of BAT thermogenesis and commencement of eating (6) was similar in the two groups (Table 2).

**Introduction of an Intruder Rat**

BAT and body temperatures of the resident rat commenced to increase promptly after the introduction of the intruder rat, whether freely moving or caged (Fig. 6, A and B). For both free and caged intruders, the slope of the initial 5 min of the BAT temperature record in the resident rat was substantially reduced in Orx_Ab rats compared with WT rats (Fig. 6, A and B, and Table 3). The amplitudes of the increase in both BAT and body temperatures (see Materials and Methods) were also substantially reduced in Orx_Ab rats.
rats for both free and caged intruder situations (Fig. 6, A and B, and Table 3).

Introduction of Live Cockroaches in a Mesh Sack

Both the initial 5-min slope of the BAT temperature record and the amplitude of the BAT temperature change were reduced in Orx_Ab rats compared with WT rats (Fig. 7 and Table 3).

After the introduction of the cockroaches, tail artery blood flow rapidly fell to near zero levels in both WT and Orx_Ab rats (Fig. 7). In Orx_Ab rats, tail artery blood flow returned to preintroduction control levels between 10 and 20 min after the cockroaches were introduced, but in WT animals, tail artery blood flow remained at low levels for a longer period (Table 3). About 2 min after the introduction of the cockroaches, the rats (WT and Orx_Ab) approached the mesh sack, clawing and biting it, but rarely actually gaining access to the cockroaches.

Acute Effect of Alerting Stimuli on Tail Artery Blood Flow

Instrumented rats were transferred from the Animal House to a closed wooden cage (40 × 40 × 40 cm, temperature: 24–26°C) fitted with a swivel device and maintained there for at least 1 h before the experimental protocol. Tail artery blood flow was continuously recorded. When flow was at a high level, four alerting stimuli (see MATERIALS AND METHODS) were administered with at least 5 min between stimuli. Figure 8 shows the original records of tail artery blood flow in an individual Orx_Ab rat (top) and an individual WT rat (bottom) before and after the administration of an alerting stimulus. For each alerting stimulus in each rat, we calculated the SCVAR index (an index of the percent fall in tail artery blood flow elicited by the alerting stimulus; see MATERIALS AND METHODS), and we combined results with the different stimuli. The combined SCVAR index was less in Orx_Ab rats compared with WT rats [70 ± 3% fall vs. 86 ± 2% fall, respectively, F(1,10) = 15.883, P = 0.003].

Comparison of BAT and Body Temperature Parameters in WT Rats in BRAC, Intruder, and Cockroach Experimental Conditions

For each experimental model (BRAC, free and caged intruder, and cockroach presentation), the slope of the initial portion of the BAT temperature record was substantially greater than the slope of the corresponding body temperature record (Table 4). In the BRAC experiments, the overall CWT
power of the 24-h BAT temperature signal was substantially greater than the corresponding value for body temperature (Table 4 and Fig. 4, B and D). For the BRAC and caged intruder experiments, the amplitude of the increase in BAT temperature was greater than the amplitude of the corresponding body temperature increase (Table 4). In the free intruder and cockroach experiments, the trend was similar, but the $P$ values were 0.067 and 0.090 (Table 4).

Table 1. Comparison of Orx_Ab and WT temperature increases associated with active phases of BRAC

<table>
<thead>
<tr>
<th>BRAC-Associated Parameters</th>
<th>Orx_Ab Group</th>
<th>WT Group</th>
<th>Factorial ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAT onset interval, min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dark</td>
<td>85 ± 2</td>
<td>94 ± 5</td>
<td>$F_{(1,28)} = 2.753, P = 0.108$</td>
</tr>
<tr>
<td>Light</td>
<td>119 ± 7</td>
<td>108 ± 7</td>
<td>$F_{(1,28)} = 0.846, P = 0.366$</td>
</tr>
<tr>
<td>BAT temperature onset values, °C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dark</td>
<td>37.4 ± 0.2</td>
<td>37.5 ± 0.2</td>
<td>$F_{(1,28)} = 0.149, P = 0.703$</td>
</tr>
<tr>
<td>Light</td>
<td>36.5 ± 0.2</td>
<td>36.5 ± 0.1</td>
<td>$F_{(1,28)} = 0.001, P = 0.994$</td>
</tr>
<tr>
<td>Body temperature onset values, °C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dark</td>
<td>38.0 ± 0.1</td>
<td>38.0 ± 0.1</td>
<td>$F_{(1,27)} = 0.130, P = 0.7208$</td>
</tr>
<tr>
<td>Light</td>
<td>37.4 ± 0.1</td>
<td>37.4 ± 0.1</td>
<td>$F_{(1,27)} = 0.004, P = 0.952$</td>
</tr>
<tr>
<td>Maximum slope of BAT temperature, °C/min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dark</td>
<td>0.09 ± 0.01</td>
<td>0.13 ± 0.01</td>
<td>$F_{(1,28)} = 17.629, P = 0.001^*$</td>
</tr>
<tr>
<td>Light</td>
<td>0.11 ± 0.01</td>
<td>0.14 ± 0.01</td>
<td>$F_{(1,28)} = 4.727, P = 0.038^*$</td>
</tr>
<tr>
<td>BAT temperature amplitude, °C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dark</td>
<td>1.26 ± 0.04</td>
<td>1.42 ± 0.06</td>
<td>$F_{(1,28)} = 5.268, P = 0.029^*$</td>
</tr>
<tr>
<td>Light</td>
<td>1.19 ± 0.06</td>
<td>1.40 ± 0.04</td>
<td>$F_{(1,28)} = 8.006, P = 0.009^*$</td>
</tr>
<tr>
<td>Body temperature amplitude, °C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dark</td>
<td>0.84 ± 0.03</td>
<td>0.96 ± 0.03</td>
<td>$F_{(1,27)} = 7.016, P = 0.013$</td>
</tr>
<tr>
<td>Light</td>
<td>0.77 ± 0.05</td>
<td>0.92 ± 0.05</td>
<td>$F_{(1,27)} = 4.555, P = 0.042^*$</td>
</tr>
<tr>
<td>BAT duration, min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dark</td>
<td>49 ± 2</td>
<td>56 ± 2</td>
<td>$F_{(1,28)} = 6.947, P = 0.014^*$</td>
</tr>
<tr>
<td>Light</td>
<td>44 ± 2</td>
<td>46 ± 2</td>
<td>$F_{(1,28)} = 0.426, P = 0.520$</td>
</tr>
<tr>
<td>BAT temperature CWT power</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dark</td>
<td>39,309 ± 2.564</td>
<td>52,590 ± 4.400</td>
<td>$F_{(1,28)} = 6.800, P = 0.015^*$</td>
</tr>
<tr>
<td>Light</td>
<td>35,096 ± 1.942</td>
<td>43,292 ± 1.711</td>
<td>$F_{(1,28)} = 10.024, P = 0.004^*$</td>
</tr>
<tr>
<td>Body temperature CWT power</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dark</td>
<td>25,033 ± 1.780</td>
<td>33,094 ± 3.011</td>
<td>$F_{(1,27)} = 5.485, P = 0.027^*$</td>
</tr>
<tr>
<td>Light</td>
<td>18,128 ± 1.505</td>
<td>20,906 ± 1.315</td>
<td>$F_{(1,27)} = 1.907, P = 0.179$</td>
</tr>
</tbody>
</table>

Values are means ± SE; $n = 15$ rats in the Orx_Ab group and 15 rats in the wild-type (WT) group. BRAC, basic rest-activity cycle; BAT, brown adipose tissue; CWT, continuous wavelet transform. *Significant difference.
and frequency of occurrence of episodic temperature increases, and the value was substantially greater for BAT temperature traces compared with body temperature.

In previous work for both BRAC and intruder models (35, 45), we demonstrated that a β3-adrenoceptor antagonist reduces the increases in both BAT and body temperatures. Similar findings have been reported in a social defeat model (29). Thus, we believe that measurement of BAT temperature with an implanted thermistor, with appropriate comparison with body temperature, provides a valid index of sympathetically controlled BAT thermogenesis and its involvement in emotional hyperthermia. We recognize that other investigators, using infrared thermography to measure BAT temperature, disagree with this view (31).

Orexin-Synthesizing Neurons Are Destroyed in Orx_Ab Rats

In our transgenic rats, we found substantial or complete loss of orexin-synthesizing perikarya in the hypothalamus and orexin-containing neuronal processes throughout the brain. This finding is similar to that reported by Beuckmann and colleagues (3) for rats of this age, but the destruction that we observed was evidently more extensive than that reported by Zhang and colleagues (65).

**BAT Thermogenesis Induced by Emotionally Significant Events Is Reduced in Orx_Ab Rats**

Body temperature increases in situations that evoke strong cognitive and emotional responses in animals, a response described as stress-induced hyperthermia or as emotional hyperthermia [references in Mohammed et al. (35)]. BAT thermogenesis contributes to emotional hyperthermia (29, 35, 53). We demonstrated that reduced BAT thermogenesis contributes to reduced body temperatures in Orx_Ab rats in our models, which evoked emotional hyperthermia by presenting a threatening or salient environmental situation. The caged intruder model uses psychological rather than physical stimuli to evoke hyperthermia. The resident rat does not directly interact with intruder and thus is not required to defend itself from an actual physical attack. Our study is the first to report that Orx_Ab animals, either rats or mice, have impaired BAT thermogenic responses to purely psychological stimuli.

Attenuated emotional hyperthermia in Orx_Ab mice in a model using insertion of a rectal probe also partially reflects BAT thermogenesis (66). Orx_Ab mice also have attenuated febrile responses to exogenous PGE2, and they are less able to maintain normothermia when exposed to a cold environment (5°C) (57). Other behavioral and autonomic responses to a threatening environment are also impaired. Mice lacking the orexin neuropeptide but with otherwise intact hypothalamic neurons have impaired cardiovascular responses, but their temperature responses are similar to WT animals (21, 57).

**Table 2. Group data of parameters related to food intake in Orx_Ab and WT rats**

<table>
<thead>
<tr>
<th></th>
<th>Orx_Ab Group</th>
<th>WT Group</th>
<th>Factorial ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amount eaten/100 g body wt in 24 h</td>
<td>4.7 ± 0.3</td>
<td>5.7 ± 0.3</td>
<td>$F_{(1,23)} = 8.044, P = 0.008^*$</td>
</tr>
<tr>
<td>Time delay before eating, min</td>
<td>18 ± 1</td>
<td>16 ± 1</td>
<td>$F_{(1,58)} = 0.644, P = 0.426$</td>
</tr>
<tr>
<td>Activity preeating in 24 h, arbitrary units</td>
<td>7 ± 2</td>
<td>12 ± 2</td>
<td>$F_{(1,58)} = 4.030, P = 0.049^*$</td>
</tr>
</tbody>
</table>

Values are means ± SE. Preeating time delay and activity were measured from the time of onset of each BRAC episode to the time of onset of eating for dark and light periods combined. *Significant difference.

Fig. 5. Averages (means ± SE) of BAT temperature records (normalized to commence at zero) for the initial 5 min after the onset of each BRAC episode during either the dark or light period. Epochs were first averaged within individual rats, and these means were then again averaged to provide an overall mean 5-min epoch for WT and Orx_Ab groups separately. SEs are shown at ~30-s intervals for clarity of presentation.
Tail Artery Vasoconstriction Induced by Emotionally Significant Events Is Reduced in Orx_Ab Rats

Salient, threatening environmental events trigger centrally programmed sympathetically mediated constriction of the tail artery (thermoregulatory cutaneous) vascular bed, preventing heat dissipation and thereby potentially contributing to emotional hyperthermia (35). In the present study, we quantified tail artery blood flow during the sudden introduction of live cockroaches into the resident rat (Orx_Ab or WT) during the 10-min preintruder period and 30-min postintruder period. For Orx_Ab rats, data are shown as solid lines with solid circles; for WT rats, data are shown as dashed lines with open circles. Each trace shows the mean ± SE, with error bars shown at 2.5 min apart. In A, the intruder rat could move freely around the cage of the resident rat. In B, the intruder rat was confined to a small cage. The start and end of the intrusion period are indicated by the thick horizontal bar.

Table 3. Comparison of Orx_Ab and WT resident rat temperature changes initiated by the introduction of an intruder rat, either freely moving or confined to a small cage, or by the introduction of a mesh sack containing live cockroaches

<table>
<thead>
<tr>
<th></th>
<th>Orx_Ab Group</th>
<th>WT Group</th>
<th>Factorial ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slope, °C/min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BAT</td>
<td>0.08 ± 0.03</td>
<td>0.20 ± 0.03</td>
<td>$F_{(1,15)} = 8.132, P = 0.012^*$</td>
</tr>
<tr>
<td>Body</td>
<td>0.05 ± 0.02</td>
<td>0.10 ± 0.01</td>
<td>$F_{(1,15)} = 4.184, P = 0.059$</td>
</tr>
<tr>
<td>Change in temperature, °C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BAT</td>
<td>0.43 ± 0.19</td>
<td>1.38 ± 0.24</td>
<td>$F_{(1,15)} = 9.573, P = 0.007^*$</td>
</tr>
<tr>
<td>Body</td>
<td>0.38 ± 0.15</td>
<td>1.00 ± 0.10</td>
<td>$F_{(1,15)} = 12.385, P = 0.003^*$</td>
</tr>
<tr>
<td>Slope, °C/min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BAT</td>
<td>0.10 ± 0.02</td>
<td>0.18 ± 0.01</td>
<td>$F_{(1,11)} = 13.363, P = 0.004^*$</td>
</tr>
<tr>
<td>Body</td>
<td>0.07 ± 0.02</td>
<td>0.10 ± 0.01</td>
<td>$F_{(1,11)} = 2.098, P = 0.175$</td>
</tr>
<tr>
<td>Change in temperature, °C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BAT</td>
<td>0.52 ± 0.14</td>
<td>1.44 ± 0.17</td>
<td>$F_{(1,11)} = 17.037, P = 0.001^*$</td>
</tr>
<tr>
<td>Body</td>
<td>0.39 ± 0.17</td>
<td>0.90 ± 0.08</td>
<td>$F_{(1,11)} = 8.075, P = 0.016^*$</td>
</tr>
<tr>
<td>Slope, °C/min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BAT</td>
<td>0.03 ± 0.02</td>
<td>0.09 ± 0.01</td>
<td>$F_{(1,14)} = 6.405, P = 0.024^*$</td>
</tr>
<tr>
<td>Body</td>
<td>0.01 ± 0.01</td>
<td>0.04 ± 0.01</td>
<td>$F_{(1,14)} = 4.096, P = 0.063$</td>
</tr>
<tr>
<td>Change in temperature, °C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BAT</td>
<td>0.15 ± 0.19</td>
<td>1.03 ± 0.08</td>
<td>$F_{(1,14)} = 18.516, P = 0.001^*$</td>
</tr>
<tr>
<td>Body</td>
<td>-0.06 ± 0.17</td>
<td>0.70 ± 0.16</td>
<td>$F_{(1,14)} = 10.880, P = 0.005^*$</td>
</tr>
<tr>
<td>Tail pulse Doppler, cm/s</td>
<td>25 ± 8</td>
<td>5 ± 2</td>
<td>$F_{(1,6)} = 6.206, P = 0.047^*$</td>
</tr>
</tbody>
</table>

Values are means ± SE. Tail artery blood flow is also shown for the cockroach experiments. *Significant difference.
cage of the resident rat. This salient event rapidly caused vigorous vasoconstriction in both WT and Orx_Ab rats. The tail artery vasoconstriction persisted for a much longer time in WT rats compared with transgenic Orx_Ab rats. Similarly, in our SCVAR experiments, we observed a greater degree of tail artery vasoconstriction in WT rats compared with Orx_Ab rats. Thus, both heat conservation via the constriction of the thermoregulatory cutaneous bed and heat production by BAT in response to salient environmental events are reduced in transgenic Orx_Ab rats.

Central Autonomic Pathways Mediating the Actions of Orexin Neurons

Central nervous system pathways mediating sympathetic control of BAT thermogenesis are reasonably well characterized (36–38, 43, 62). Central nervous system pathways mediating sympathetic control of the tail artery vasculature have also been studied (7, 8, 12, 34, 40–42, 56, 58, 59, 63). Roles for orexin-synthesizing neurons in arousal, in food intake and metabolism, and in the integration of behavioral and autonomic responses to environmental threat and to arousal and appetitive functions have also been demonstrated in other experimental models (11, 19, 20, 22, 26, 50, 52, 60, 61). Neural pathways involved in these functions include the amygdala, dorsomedial hypothalamus, locus coeruleus, and medullary raphe (2, 4, 46, 48, 52, 68). Orexin-synthesizing neurons innervate all these regions, and orexin receptors have also been demonstrated in these autonomic centers (30).

BRAC Organization of Daily Life Is Intact in Orx_Ab Rats, But BAT Thermogenesis Is Reduced During Active BRAC Phases

Our present study is the first to examine BRAC in Orx_Ab rats. The BRAC organization, with periods of activity and food intake interspersed with periods of rest, is preserved in transgenic Orx_Ab animals. There were no differences between transgenic Orx_Ab and WT rats in the time intervals between active BRAC episodes, in either 12-h dark or 12-h light phases of the daily cycle. We have previously discussed the irregularity (nonstationarity) of the timing of BRAC episodes, a feature of ultradian rhythms (5, 6, 44).

The ~15-min delay between the onset of an active BRAC episode and the commencement of eating was also similar in transgenic Orx_Ab and WT animals. Although eating occurs during BRAC episodes, the ultradian organization of daily life continues substantially unchanged in rats deprived of food (44), and thus the central ultradian pattern generator (1) is intact in Orx_Ab rats. So far, studies in Orx_Ab mice have not included analysis of the BRAC organization in this species.

Fig. 7. Grouped data showing averaged original records of BAT and body temperatures and tail artery blood flow in the resident rat (Orx_Ab or WT) pre- and postintroduction of cockroaches. For Orx_Ab rats, data are shown as solid lines with solid circles; for WT rats, data are shown as dashed lines with open circles. Each trace shows the mean ± SE, with error bars shown at ~2.5 min apart. The start and end of the cockroach intrusion period are indicated by small arrows at the timescale. The values for tail artery blood flow are taken at 15 min after cockroach introduction.

Fig. 8. Pulsatile tail artery blood flow signal recorded during a single tap (indicated by small arrows) on the side of the closed wooden cage in an individual transgenic Orx_Ab rat (rat Orx_Ab001; top) and in an individual WT rat (rat WT005; bottom).
We have previously demonstrated that episodic increases in body and brain temperature, occurring approximately every 90 min during the dark active period as part of BRAC, are partially due to BAT thermogenesis (44). At the beginning of an active BRAC episode, in both dark and light phases of the daily cycle, resting BAT and body temperatures in Orx_Ab rats are the same as resting temperatures in WT rats. As animals explore the external environment in preparation for eating, BAT temperature increases in both groups of rats, but the rate and amplitude of the increase are much greater in WT rats. Thus, during a BRAC episode, body temperature increases less in Orx_Ab rats than in WT rats, and this is at least partially because of substantially reduced BAT thermogenesis in transgenic Orx_Ab rats. This BRAC-associated difference between WT and transgenic Orx_Ab animals is more marked in the dark phase, when the animals are generally more actively interacting with the environment.

BRAC is thus an important framework for comparisons of Orx_Ab and WT rats. A previous study (51) of Orx_Ab rats did not observe significant differences in body temperature between Orx_Ab and control WT rats, but that study did not differentiate between active and inactive phases of BRAC. Similarly, in our present study, the observed significant differences were not detected when the analysis was performed without taking account of BRAC (M. Mohammed, Y. Ootsuka, and W. Blessing, unpublished observations).

The BRAC-related increases in temperature occur “spontaneously,” as part of normal daily life. They are not evoked by “stress” or “defence.” Thus, it is the behavioral and autonomic functions normally engaged during active exploration of the environment that are muted in Orx_Ab rats.

Even though our Orx_Ab rats had virtually complete loss of orexin-synthesizing neurons, baseline core body temperature was not reduced, and the temperature still increased in response to an intruder rat and during BRAC episodes. Our data suggest that orexin-synthesizing neurons amplify behavioral and autonomic functions associated with interactions with the environment. The neurons are not essential components of the neural circuitry mediating these interactions.

### Perspectives and Significance

The term “orexin” was introduced by Sakurai and colleagues (49) because it suggested increased appetite for food. In view of the more general functions now known to be amplified by this neuropeptide, it is interesting to note that Aristotle introduced the word “orexis” to describe appetite/desire in a more general sense, not just the appetite for food (54). The term suggests the initiation of goal-directed interactions with the environment. We (44) have previously documented that the onset of BRAC episodes is signaled by the sudden increase in the proportion of theta (8–12 Hz) rhythms in the hippocampal electroencephalogram. Theta rhythm is a marker for cognitive processes engaged during active attention to the external environment (9, 10), and these processes are associated with increases in brain temperature (23, 55). We have hypothesized that the centrally programmed BAT thermogenesis initiated during an interaction with the external environment contributes to this brain temperature increase and thereby facilitates the neural events underlying cognitive processing (5, 6, 35, 44).

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### DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

### AUTHOR CONTRIBUTIONS

Author contributions: M.M., Y.O., and W.B. conception and design of research; M.M., Y.O., and W.B. performed experiments; M.M., Y.O., and W.B. analyzed data; M.M., Y.O., and W.B. interpreted results of experiments; M.M., Y.O., and W.B. prepared figures; M.M., Y.O., and W.B. drafted manuscript; M.M., Y.O., and W.B. edited and revised manuscript; M.M., Y.O., M.Y., and W.B. approved final version of manuscript.
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