An Orexinergic Projection from Perifornical Hypothalamus to Raphe Pallidus Increases Rat Brown Adipose Tissue Thermogenesis

Domenico Tupone,1 Christopher J. Madden,1 Georgina Cano,2 and Shaun F. Morrison3
1Department of Neurological Surgery, Oregon Health & Science University, Portland, Oregon 97239-3098, and 2Department of Neuroscience, University of Pittsburgh, Pittsburgh, Pennsylvania 15260

Orexin (hypocretin) neurons, located exclusively in the PeF-LH, which includes the perifornical area (PeF), the lateral hypothalamus (LH), and lateral portions of the medial hypothalamus, have widespread projections and influence many physiological functions, including the autonomic regulation of body temperature and energy metabolism. Narcolepsy is characterized by the loss of orexin neurons and by disrupted sleep, but also by dysregulation of body temperature and by a strong tendency for obesity. Heat production (thermogenesis) in brown adipose tissue (BAT) contributes to the maintenance of body temperature and, through energy consumption, to body weight regulation. We identified a neural substrate for the influence of orexin neurons on BAT thermogenesis in rat. Nanoinjection of orexin-A (12 pmol) into the rostral raphe pallidus (rRPa), the site of BAT sympathetic premotor neurons, produced large, sustained increases in BAT sympathetic outflow and in BAT thermogenesis. Activation of neurons in the PeF-LH also enhanced BAT thermogenesis over a long time course. Combining viral retrograde tracing from BAT, or cholera toxin subunit b tracing from rRPa, with orexin immunohistochemistry revealed synaptic connections to BAT from orexin neurons in PeF-LH and from rRPa neurons with closely apposed, varicose orexin fibers, as well as a direct, orexinergic projection from PeF-LH to rRPa. These results indicate a potent modulation of BAT thermogenesis by orexin released from the terminals of orexin neurons in PeF-LH directly into the rRPa and provide a potential mechanism contributing to the disrupted regulation of body temperature and energy metabolism in the absence of orexin.

Introduction
Orexins (hypocretins) (de Lecea et al., 1998; Sakurai et al., 1998) are neuropeptides synthesized in the brain exclusively by neurons in the PeF-LH, an area including the lateral hypothalamus (LH), perifornical area (PeF), and lateral parts of the medial hypothalamus. Orexinergic neurons have widespread projections and influence a variety of physiological functions including sleep–wake states and stress-arousal responses (Sakurai et al., 2010; Mieda et al., 2011), and neuroendocrine and autonomic effects (Machado et al., 2002; Smith et al., 2010; Kuwaki, 2011). Loss of orexin neurons from the PeF-LH leads to the disordered sleep patterns of narcolepsy, but is often accompanied by defective energy and metabolic homeostasis, including a high risk for obesity (Kok et al., 2003; Hara et al., 2005) and the potential for altered thermoregulation (Plazzi et al., 2011). The Orx-A isoform has a high affinity for both orexin receptor subtypes (Orx1R and Orx2R) (Sakurai et al., 1998).
orexin neurons on BAT thermogenesis and energy expenditure. Conventional and viral retrograde tracing techniques revealed a direct orexinergic projection from PeF-LH to rRPa, targeting rRPa neurons synthetically connected to BAT. Functionally, activation of neurons in PeF-LH or nano-injections of Orx-A directly into rRPa elicited a marked and prolonged enhancement of BAT sympathetic outflow and BAT thermogenesis. These results indicate a potent thermogenic role for orexin released from the terminals of PeF-LH neurons to directly activate neurons in the rRPa, likely the local BAT sympathetic premotor neurons.

Materials and Methods

Experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals, Eighth Edition (National Research Council, National Academies Press, 2010) and protocols were approved by the Institutional Animal Care and Use Committees of Oregon Health and Science University and of the University of Pittsburgh.

Anatomical studies

Viral injections and immunohistochemical procedures. Adult male, Sprague Dawley rats (275–350 g, Zivic-Miller Laboratories) were maintained in a 12 h, light/dark cycle (lights on at 7:00 A.M.) with ad libitum access to food and water. Rats were housed in a Biosafety level 2 facility throughout the experiments and their health was carefully monitored after viral injections.

Rats were anesthetized with an intraperitoneal injection of ketamine (60 mg/kg) and xylazine (7 mg/kg). The interscapular BAT was exposed via a 2.5 cm incision along the back, and 0.1 µl of an attenuated strain of Pseudorabies virus [PRV-Bartha, 4 × 10^8 plaque-forming units (pfu)/ml] was injected into 5 different sites in a circular distribution (total volume = 0.5 µl), using a 1 µl Hamilton syringe. After injections, the skin was closed with wound clips. Different postinoculation times were used to estimate the temporal progression of the virus through the CNS after injection into BAT: 78 h (2 rats), 84 h (1 rat), 90 h (2 rats) and 96 h (2 rats). A second group of rats was injected with PRV-BaBlu (4 × 10^8 pfu/ml), an isoergic recombinant of PRV-Bartha that contains the LAC Z gene encoding for β-galactosidase under the control of the viral promoter. As PRV-Bartha, PRV-BaBlu is transported retrogradely through polysynaptic circuits after injection in a specific target (Standish et al., 1995; Elmslie and Saper, 1996; Card and Enquist, 1999; Billig et al., 2000). Following a similar procedure as described above, a total volume of 1 µl of PRV-BaBlu (5 injections of 0.2 µl) was injected into BAT in 6 rats. The inoculation times used were: 84 h (3 rats), 96 h (2 rats), and 102 h (1 rat). After the appropriate post-inoculation time, rats were deeply anesthetized with ketamine (120 mg/kg) and xylazine (14 mg/kg) and were perfused transcardially with 0.9% saline followed by paraformaldehyde-lvines-periodate fixative. The brains were after fixed for 3 h and preserved in 25% sucrose at 4°C. Serial coronal sections (35 µm) were cut with a freezing-stage microtome, collected sequentially in 8 sets and stored in cryoprotectant solution at −20°C before immunohistochemical processing. The tissue samples from rats injected with PRV-Bartha used in this study are a subset of infection cases from a previous study in which the temporal distribution of PRV-infected neurons in the CNS after BAT injection was described in detail (Cano et al., 2003).

A dual fluorescence labeling procedure was performed in one set of brain sections from each rat to characterize PRV-positive (Bartha or BaBlu) and orexin-positive neurons. Neurons infected with PRV-Bartha were identified using a rabbit polyclonal antisera (Rb 133, 1:2000) that recognizes viral envelope and capsid proteins (Card and Enquist, 1994). Neurons infected with PRV-BaBlu were labeled using a mouse anti-β-galactosidase antibody (max β-gal, Sigma, 1:1000). A goat polyclonal antibody against Orx-A (goOrx, Santa Cruz Biotechnology, 1:1000) was used to identify orexinergic neurons in the PeF-LH. Brain sections were pretreated with 0.5% sodium borohydride in 10 mM sodium phosphate buffer (PBS) during 30 min to reduce protein cross-linking caused by aldehyde fixation. Subsequently, sections were incubated for 1 h at room temperature followed by 48 h at 4°C in a mixture of two primary antibodies (goOrx and Rb 133 for PRV-Bartha injections, and goOrx and mα β-gal for PRV-BaBlu injections) diluted in PBS containing 1% normal donkey serum and 0.3% Triton X-100. Then, sections were incubated for 2 h in the dark at room temperature in a combination of two secondary antibodies: Alexa Fluor 555-conjugated donkey anti-goat IgG (1:500; Invitrogen) to label the cytoplasm of Orx-A-containing neurons and projections in red and DyLight 488-conjugated donkey anti-rabbit IgG (1:500; Jackson ImmunoResearch Laboratories) or Alexa Fluor 488-conjugated donkey anti-mouse IgG (1:500; Invitrogen) to label the nucleus (early infection) or the nucleus and cytoplasm of PRV-Bartha- and PRV-BaBlu-infected neurons, respectively, in green. Double-labeled, PRV-infected, Orx-A-immunoreactive (Orx-A-ir) neurons displayed yellow fluorescence in the cytoplasm. All steps were preceded by several washes in PBS. Subsequently, sections were mounted, air-dried, dehydrated and cleared in xylene and coverslipped.

Tissue analysis. The neuroanatomical location of PRV-infected neurons in rRPa, parapyramidal area (PaPy), and of Orx-A-ir neurons in the PeF-LH was based on the stereotaxic rat brain atlas of Paxinos and Watson (Paxinos and Watson, 2007). PRV-infected neurons (non-Orx-A-ir) and PRV-infected, Orx-A-ir neurons (dual-labeled neurons) were counted in the PeF-LH bilaterally in 5 consecutive sections (from a 1.8 set of sections; 245 µm distance between consecutive sections). The percentages of PRV-infected, Orx-A-ir neurons relative to the total number of infected neurons in the entire PeF-LH were calculated for each rat. Cases were grouped based on the total number of PRV-infected neurons in the PeF-LH (see Results) and the mean ± SEM were calculated for each group. Photomicrographs of PRV-infected neurons and Orx-A-ir neurons and fibers were obtained using an image capture system (Simple PCI, version 6.6, Hamamatsu Corp.) attached to an Olympus BX51 fluorescence microscope. The photomicrographs were assembled into a plate using Adobe Photoshop to adjust contrast and brightness without altering the original colors.

CTb injections in rRPa and PaPy and immunohistochemical procedures. Four adult male, Sprague Dawley rats (240 – 400 g), anesthetized with 3% isoflurane in 100% O2, were stereotaxically injected with cholera toxin subunit b (CTb, 1 mg/ml, 120 nl) conjugated to Alexa488 either into rRPa (n = 2) or PaPy (n = 2); coordinates with incisor bar at −11 mm: 3.0 mm rostral, 1.1 mm lateral, and 3.0 mm ventral to calamus scriptorius) and the pipette was left in place for 3 min. Rats were treated with antibiotic (40,000 U/kg penicillin G, i.m.), analgesic (0.05 mg/kg buprenorphine, i.m.) and saline (3 ml, s.c.). After 7 d, the rats were deeply anesthetized with pentobarbital (80 mg/kg, i.p.) and transcardially perfused with saline followed by 4% paraformaldehyde. The brains were postfixed in 4% paraformaldehyde for 1–2 h and equilibrated overnight in PBS with 20% sucrose and 0.01% sodium azide. Serial coronal sections (30 µm) were cut with a freezing-stage microtome, collected sequentially in 5 sets and stored in PBS with 0.01% sodium azide at 4°C.

Sections containing PeF-LH were preincubated in an antibody dilution solution (ADS: 500 ml of PBS, 0.3% Triton X-100, 1.25 g of carrageenan, 100 mg of NaN3, 5 ml of normal donkey serum) for 3 h, pretreated with avidin-biotin blocking kit (SP-2001, Vector) and incubated overnight at room temperature with the primary antibodies for Orx-A (1:5000, rabbit-anti-orexin-A, PC362, Calbiochem) and CTb (1: 20,000, goat-anti-CTb, 703, List Biological). After two washes in PBS containing 0.3% Triton X-100 (TPBS, 20 ml), the tissue was incubated for 1 h in ADS containing in the secondary antibody for Orx-A (1:500, biotin-SP-donkey-anti-rabbit, Jackson ImmunoResearch Laboratories). After two washes in TPBS, the tissue was incubated for 1 h in ADS containing the secondary antibody for CTb (10 µg/ml, Alexa Fluor 488-donkey-anti-goat, Invitrogen) and streptavidin conjugated to Alexa Fluor 594 (3–5 µg/ml, Invitrogen). After fluorescent labeling, the tissue was washed in PBS and mounted onto coated slides, air dried and coverslipped with anti-fade mounting medium (Pro-Long Gold, Invitrogen).

Tissue analysis. The neuroanatomical designations of the injection sites in rRPa and PaPy and those of CTb- and Orx-A-labeled neurons in the PeF-LH are based on the stereotaxic rat brain atlas of Paxinos and Watson (Paxinos and Watson, 2007). Photomicrographs of brain sections and labeled neurons were taken with a camera attached to an Olympus BX51 fluorochrome microscope. The photomicrographs were

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assembled into a plate using Adobe Photoshop to adjust contrast and brightness without altering the original colors.

**Physiological experiments**

**Surgical procedures.** Male Wistar rats (300–400 g, Charles River, n = 30) were maintained in a 12 h, light/dark cycle (light on at 7:00 A.M.) with ad libitum access to food and water. Rats were anesthetized initially with 3% isoflurane in 100% O2 and transitioned to urethane (0.8 g/kg) and chloralose (80 mg/kg) following cannulation of a femoral artery and vein. Heart rate (HR) was derived from the arterial pressure signal. After the animals were positioned, a femoral artery and vein were cannulated for blood pressure and fluid infusion. A subcutaneous infusion catheter was placed in one ear and connected to a bolus syringe. The animals were then secured to a stereotaxic frame and a 25-gauge needle was inserted into the left iBAT pad through the back skin and a midline incision through the back skin and a midline incision. The needle was then advanced to the site of interest, and its position confirmed by skin temperature measurement. The needle was then withdrawn, and fluid meniscus in the micropipette. To make multiple nanoinjections at the same site, the micropipette was retracted vertically, emptied, rinsed with saline, refilled and then repositioned to the original dorsoventral coordinate. The microinjection sites were marked by pressure microinjection of fluorescent polystyrene microspheres (1:10 dilution of Fluospheres, F8797, F8801 or F8803, Invitrogen). After physiological recordings, rats were perfused transcardially with 0.9% isotonic saline, except SB-334867 that was dissolved in dimethyl sulfoxide (DMSO). Orx-A was chosen for these studies based on its high

**Nanoinjections.** Glass micropipettes (outer tip diameter, 20–30 μm) were used for all drug nanoinjections which were given over a 5–10 s, using a pressure injection system (model Ue, Toohey Company). Nanoinjection volumes (60 nl) were determined from the displacement of the fluid meniscus in the micropipette. To make multiple nanoinjections at the same site, the micropipette was retracted vertically, emptied, rinsed with saline, refilled and then repositioned to the original dorsoventral coordinate. The microinjection sites were marked by pressure microinjection of fluorescent polystyrene microspheres (1:10 dilution of Fluospheres, F8797, F8801 or F8803, Invitrogen). After physiological recordings, rats were perfused transcardially with 0.9% isotonic saline, except SB-334867 that was dissolved in dimethyl sulfoxide (DMSO). Orx-A was chosen for these studies based on its high

**Table 1. PRV-infected neurons and orexin-immunoreactive neurons in PeF-LH**

| Infection group | Number of PRV-infected neurons in PeF-LH | Number of rats | Orexin + PRV / total PRV |
|-----------------|-----------------------------------------|---------------|--------------------------|
| Light infection | 1–4                                     | 2             | 0%                       |
|                 | 2–8                                     | 3             | 44.0 ± 5.5%              |
| Intermediate infection | 10–65                                  | 5             | 42.0 ± 10.2%             |
| High infection  | 120–180                                 | 3             | 21.0 ± 0.4%              |

**Drugs.** Orexin-A, N-methyl-ω-aspapartate (NMDA) and the orexin agonist, SB-334867, were obtained from Tocris Bioscience and dissolved in isotonic saline, except SB-334867 that was dissolved in dimethyl sulfoxide (DMSO) and diluted in isotonic saline to a final concentration of <10% DMSO. Orex-A was chosen for these studies based on its high affinity for both Orx1-R and Orx2-R and the dose was based on a previous study using direct intraparenchymal injections (Chen et al., 2000). The
concentration of SB-334867 was based upon the effective dose of orexin, the relative affinities for orexin receptors and the previous observation that the concentration of SB-334867 required to block (>90% inhibition) Orx-A-evoked Ca\(^{2+}\) entry was 100-fold higher than that of Orx-A (Smart et al., 2001).

**Experimental protocols**

**Experiment 1: injection of Orx-A in rRPa.** Rats (n = 7) receiving a nanoinjection of Orx-A (12 pmol) into rRPa at baseline conditions in which \(T_{\text{Core}}\) was maintained at a temperature slightly below 37°C, which produced a low level of basal BAT SNA.

**Experiment 2: injection of Orx-A in the PaPy.** Rats (n = 4) received a nanoinjection of Orx-A (12 pmol) into PaPy (left side; coordinates: 3.0 mm caudal to lambda, 1.2 mm lateral to the midline, and 9.5–9.8 mm ventral to the dura) under the same baseline cool conditions as in Experiment 1.

**Experiment 3: injection of Orx-A in rRPa in warm animals.** Rats (n = 4) received a nanoinjection of Orx-A (12 pmol) into rRPa while \(T_{\text{Core}}\) was maintained at a sufficiently warm temperature (>37°C) to ensure the absence of spontaneous BAT SNA.

**Experiment 4: injection of NMDA in PeF-LH.** Rats (n = 6) received a nanoinjection of NMDA (12 pmol) into the PeF-LH (coordinates:...
provide an estimation of the relative sequence of retrograde infections of neurons in these two regions.

The pattern of infection in the CNS was reproducible and almost identical after PRV-Bartha or PRV-BaBlu injections into BAT. Since both viruses are retrogradely transported only through synaptically linked neurons, the infected brain regions are most likely those involved in the control of sympathetic outflow to BAT. As in our earlier detailed description of PRV-Bartha infection in the CNS after BAT injection (Cano et al., 2003), infection was first detected in the sympathetic preganglionic neurons (SPNs) located in the intermediolateral cell column of the upper thoracic cord at early post-inoculation times. At longer times, the infection progressed within the spinal cord and into the brain, where infected neurons were observed in sympathetic premotor areas, including the medullary raphe nuclei, containing the rRPas. These brain neurons most likely became infected via direct projections to infected SPNs.

At short post-inoculation times, a few infected neurons were observed in the intermediate extent of the caudal raphe nucleus, mainly in the RMg and rRPas. At intermediate post-inoculation times, infected raphe neurons were found between −10.30 to −12.30 mm from bregma (Paxinos and Watson, 2007), being most numerous between −11.00 and −11.60 mm from bregma (Fig. 1A, B). Rostrally, PRV-infected neurons were found in the RMg, rRPas and PaPy; more caudally, PRV-infected neurons were
observed in the rRPa, raphe obscurus (ROb) and PaPy, although the number of infected neurons was much lower than in rostral sections. At longer post-inoculation times, PRV-infected neurons were observed in all sections throughout the rostrocaudal extent of the medullary raphe nuclei.

Dual fluorescence labeling suggested that PRV-infected neurons in the rRPa and PaPy were heavily innervated by orexin fibers (Fig. 1A, B): apposition-like structures were observed at high magnification in both regions but appeared denser in the PaPy (Fig. 1A). At short post-inoculation times, when only sympathetic premotor groups would have been infected, orexin fibers were observed in close apposition to infected neurons in rRPa and PaPy, consistent with orexin fibers innervating the rRPa and PaPy that directly project to the spinal cord. At longer post-inoculation times that allowed another viral replication cycle to occur, orexin fibers apposing infected neurons in rRPa and PaPy (Fig. 1A–C) were most likely innervating interneurons within the raphe nuclei that became infected via their local inputs to infected BAT sympathetic premotor neurons. Although varicose orexin fibers in close apposition to infected rRPa and PaPy neurons have been previously reported after PRV injection into BAT (Berthoud et al., 2005), the large extent of infection in this study precluded their opportunity to suggest that such orexin fibers may have directly innervated BAT sympathetic premotor neurons.

PRV-infected neurons in the PeF-LH appeared later than those in the mediullary raphe nuclei and reflected projections to previously infected brain regions, such as rRPa and PaPy, known to be efferent targets of orexin fibers (Peyron et al., 1998; Berthoud et al., 2005), or through the very sparse projections from PeF-LH to spinal SPNs (Llewellyn-Smith et al., 2003). PRV infection did not impair orexin synthesis and expression in infected neurons since dual-labeled neurons were observed in the PeF-LH at late post-inoculation times. PRV-infected orexin neurons did not show a specific topographic distribution and were found scattered in the entire PeF-LH. Nevertheless, they were more commonly observed dorsolateral to the fornix (Fig. 1D, E) and, to a lesser extent, in the ventrolateral part of LH close to the optic tract (Fig. 1F).

At early stages of viral infection in a neuron, the virus is only detected in the nucleus and as the infection progresses the viral particles or marker (β-gal in the case of PRV-Bartha) are detected in both the nucleus and cytoplasm. For cell counting, neurons were considered to be infected when either the nucleus or the nucleus and cytoplasm were labeled. Similarly, dual-labeled, PRV-infected, Orx-A-ir neurons were counted when only the nucleus was infected (green nucleus, red cytoplasm) and when both nucleus and cytoplasm were infected (green nucleus, yellow cytoplasm) (Fig. 1F).

In general, the number of PRV-infected neurons (orexin and non-orexin) in PeF-LH increased at longer post-inoculation times. However, some variability in the extent of infection was found among samples within the same post-inoculation interval (this variability might be due to immobilization of the virus inside the fat, which may take longer for the viral particles to reach the axon terminals from sympathetic ganglion neurons). For this reason, we calculated the percentage of PRV-infected neurons that were orexin-positive by grouping the cases on the basis of the total number of infected neurons in the PeF-LH rather than strictly by post-inoculation time. Since there were no differences in the pattern and extent of infection between PRV-Bartha- and PRV-BaBlu-infected animals, these cases were grouped together. The cell counting results for the following groups are reported in Table 1: light infection (rats with <10 PRV-infected neurons in PeF-LH, n = 5), intermediate infection (rats with 10–65 PRV-infected neurons in PeF-LH, n = 5) and high infection (rats with 120–180 PRV-infected neurons in PeF-LH, n = 3).

The variability among cases in the light and intermediate infection groups was high, most likely because of the lower total number of infected neurons and because of a sampling effect (we counted neurons in a 1:8 set of sections). In contrast, the variability was very low in the highly infected group, suggesting that the percentage obtained (~20%) might be a good estimate of the real percentage of orexin neurons with respect to total PeF-LH neurons that are involved in the central control of sympathetic out-
flow to BAT. This is in agreement with a report (Oldfield et al., 2002) that after PRV injection into BAT, 32% of PRV-infected neurons in the LH were orexin-positive, although direct comparison with our results is precluded by the absence of information on post-inoculation times and on the number of rats and sections per rat that were quantified in their analysis.

**CTb injection into rRPa and PaPy**

To determine the distribution of the orexinergic neurons in PeF-LH projecting specifically to the rRPs and to the PaPy, we injected the retrograde tracer, CTb into either rRPs or PaPy and then examined the PeF-LH for neurons containing CTb and Orx-A. Almost all of the cells that were double-labeled for CTb and Orx-A (Fig. 2Aii) following nanoinjection of CTb into the rRPs (Fig. 2Aiii) were found in the PeF-LH region between bregma: −3.0 and −3.5 mm (Fig. 2Ai). Overall, the distribution of double-labeled, CTb- and Orx-A-ir neurons (Fig. 2Bii) following CTb injection in PaPy (Fig. 2Biii) was similar to that following CTb injection into the rRPa: most of the Orx-A-ir neurons projecting to PaPy were found in the PeF-LH at levels between bregma: −3.0 and −3.5 mm (Fig. 2Bi) and they were distributed both ipsilaterally and contralaterally to the injected PaPy. Whereas the double-labeled neurons in PeF-LH following CTb injection into the midline rRPs were evenly distributed bilaterally and rostrocaudally, those following CTb injection into the PaPy were distributed relatively more on the side ipsilateral to the injected PaPy and more rostrally in the PeF-LH (i.e., section bregma: −3.00 mm, Fig. 2Bi). Also noteworthy, is the similarity in the retrograde labeling from the two mediatory injection sites of non-Orx-A-ir neurons in the dorsomedial hypothalamus (DMH) and dorsal hypothalamic area (compare localization of CTb-only neurons in bregma −3.24 sections in Fig. 2Aii,Bii). There was also a relative absence of retrogradely labeled, non-Orx-A-ir neurons in the caudal level of the PeF-LH (section at bregma −3.48) following CTb injections in PaPy compared with the larger number of such neurons in the PeF-LH at this level following CTb injection into rRPs.

**Injection of Orx-A into rRPa and or PaPy**

To assess the potential functional significance of the finding of a dense network of varicose orexin fibers surrounding neurons in rRPs and PaPy infected following PRV injections into BAT (Fig. 1A–C), we determined the effect of nanoinjection of Orx-A in rRPs or PaPy on BAT SNA and BAT thermogenesis. Experiments were performed under two conditions: first, TCore was maintained at levels, always <37°C, that produced a low level of baseline BAT SNA and second, TCore was maintained at levels, usually >37°C, that eliminated any baseline BAT SNA. Modest skin and core cooling (TCore 35.9 ± 0.1°C) produced a level of baseline SNA that was 257 ± 35% of the control level recorded when the rats were warmed and BAT SNA was absent. Under these cool conditions, nanoinjection of Orx-A (12pmol) into rRPs (Fig. 3C,D) produced a rapid, strong and sustained (>1 h) increase in BAT SNA and in BAT thermogenesis (Fig. 3A,B). BAT SNA increased (p < 0.05, n = 6) by 95%, from 257 ± 35% of control before Orx-A nanoinjection to a peak of 500 ± 87% of control at 30 min following Orx-A nanoinjection into rRPs, and produced a rise (p < 0.05; n = 7) in TBAT from 31.9 ± 0.4°C at baseline to a peak of 33.7 ± 0.5°C. The Orx-A-evoked stimulation of BAT thermogenesis contributed to an increase (p < 0.05; n = 7) in expired CO2 from 3.0 ± 0.1% at baseline to a peak of 3.3 ± 0.1% and in TCore from 35.9 ± 0.1°C to a peak of 36.2 ± 0.2°C (p < 0.05; n = 7). Orx-A nanoinjection into rRPs increased HR from 412 ± 17 bpm to a peak of 443 ± 9 bpm (p < 0.05; n = 7), but did not change arterial pressure (Fig. 3A,B).

Under the cool conditions, nanoinjection of Orx-A into PaPy (Fig. 4C,D) elicited a similar long lasting (>1 h) increase (p < 0.05, n = 4) in BAT SNA from 384 ± 162% of control at baseline to a peak of 597 ± 127% of control (Fig. 4B, top trace) that resulted in a rise (p < 0.05) in BAT thermogenesis from a TBAT of 33.2 ± 0.7°C at baseline to a peak of 34.6°C ± 0.6. Simultaneously, expired CO2 rose (p < 0.05) from 3.2 ± 0.5% at baseline to 3.6 ± 0.6% peak, and TCore increased (p < 0.05) from 36.3 ± 0.4°C at baseline to a peak of 36.7 ± 0.4°C. Orx-A nanoinjection into PaPy evoked a tachycardia in which HR rose (p < 0.05) from 444 ± 11 bpm at baseline to a peak of 470 ± 9 bpm (Fig. 4A,B).

![Figure 5. A. Under the baseline condition of warmed skin and core temperatures, which eliminated BAT SNA, nanoinjection of Orx-A (dashed line) in rRPa did not evoke any change in BAT SNA, TBAT, expired CO2, TCore, HR, or mean arterial pressure (MAP). The means ± SEM (n = 4) of the time courses of the physiological variables (points are 30 s averages of the variable values) during the nanoinjection of Orx-A into rRPa are shown in B, C. Histological section shows the Orx-A nanoinjection site in rRPa (white arrowhead) for the responses illustrated in A, B. The distribution of Orx-A nanoinjection sites are plotted on an atlas drawing (Paxinos and Watson, 2007) containing the rRPa at the level of the facial nucleus (7n). Py, Pyramidal tract.](image-url)
BAT thermogenesis, that there are orexinergic neurons in PeF-LH that are synaptically connected to BAT and that some orexinergic neurons in the PeF-LH project directly to rRPa, we next tested the hypothesis that activation of neurons in the PeF-LH would activate BAT SNA and BAT thermogenesis in a manner similar to that evoked by Orx-A nanoinjection into rRPa and PaPy. As in the experiment with Orx-A injections into rRPa, the BAT responses to activation of neurons in PeF-LH were examined under cool conditions with a low level of ongoing BAT SNA and under warm conditions, where BAT SNA was absent. When Ts, and T were lowered to generate a low level of BAT SNA, nanoinjection of NMDA to excite local neurons, including orexin neurons, in PeF-LH (Fig. 6C,D) produced a marked and sustained increase in BAT SNA (Fig. 6A,B) that was largely similar to that following nanoinjection of Orx-A into rRPa, except for the addition of a distinct, rapid, early phase to the rise in BAT SNA (Fig. 6A,B). During this initial phase (−4 min duration) of the increase in BAT SNA evoked by NMDA nanoinjection into PeF-LH, BAT SNA rose rapidly to a peak level that was 180% greater than that before the NMDA injection (baseline: 263 ± 33% of control vs peak: 736 ± 132% of control, p < 0.05, n = 6) and that was significantly greater (p < 0.05, n = 5, one outlier lacking a distinct early, rapid increase in BAT SNA was excluded) than the level of BAT SNA reached during the same period following Orx-A nanoinjection into rRPa (n = 6). There was no difference (p > 0.05) between the baseline levels of BAT SNA for these two groups.

After the subsidence of the early increase following NMDA nanoinjection into PeF-LH, the increase in BAT SNA was sustained for >1 h and reached a peak increase of 108% (baseline: 263 ± 33% of control vs peak: 547 ± 90% of control, p < 0.05, n = 6), which was not different (p > 0.05) from the sustained increase in BAT SNA following Orx-A nanoinjection into rRPa. The BAT thermogenic response to nanoinjection of NMDA into PeF-LH consisted of an increase in T BAT from 32.7 ± 0.4°C at baseline to a peak of 33.8°C ± 0.5°C (p < 0.05, n = 6), which contributed to the increase in expired CO2 (baseline: 3.7 ± 0.2% vs peak: 4.0 ± 0.2% peak, p < 0.05, n = 6) and in Tcore (36.2 ± 0.2°C baseline vs 36.7 ± 0.2°C peak; p < 0.05; n = 6) (Fig. 6A,B). Activation of neurons in PeF-LH with NMDA also evoked a brief (~2 min) fall in arterial pressure (94 ± 6 mmHg baseline vs 79 ± 7 mmHg nadir, p < 0.05, n = 6) immediately following the NMDA injection in PeF-LH (Fig. 6A,B), which did not occur following Orx-A nanoinjection into rRPa (Fig. 3A,B).

Injection of Orx-A into rRPa under warm conditions
Since these data demonstrated the ability of Orx-A to potentiate an ongoing sympathetically mediated BAT thermogenesis, we next tested whether Orx-A alone, would be sufficient to drive BAT SNA and BAT thermogenesis in the absence of basal BAT SNA. Nanoinjection of Orx-A in rRPa in rats whose Tcore was maintained at a level necessary to completely silence spontaneous BAT SNA (usually 37–38°C). Under these warm conditions, nanoinjection of Orx-A into rRPa (Fig. 5C,D) did not elicit a change in any of the recorded variables (Fig. 5A,B). In 3 of 4 cases, several bursts of BAT SNA were present immediately following the Orx-A injection (Fig. 5A, second trace), however, these were sparse, short-lived and highly variable in their occurrence.

Nanoinjection of NMDA into PeF-LH stimulates BAT thermogenesis
Having demonstrated that orexin can act in the rRPa to stimulate BAT thermogenesis, that there are orexinergic neurons in PeF-LH that are synaptically connected to BAT and that some orexinergic neurons in the PeF-LH project directly to rRPa, we next tested the hypothesis that activation of neurons in the PeF-LH would activate BAT SNA and BAT thermogenesis in a manner similar to that evoked by Orx-A nanoinjection into rRPa and PaPy. As in the experiment with Orx-A injections into rRPa, the BAT responses to activation of neurons in PeF-LH were examined under cool conditions with a low level of ongoing BAT SNA and under warm conditions, where BAT SNA was absent. When Ts, and T were lowered to generate a low level of BAT SNA, nanoinjection of NMDA to excite local neurons, including orexin neurons, in PeF-LH (Fig. 6C,D) produced a marked and sustained increase in BAT SNA (Fig. 6A,B) that was largely similar to that following nanoinjection of Orx-A into rRPa, except for the addition of a distinct, rapid, early phase to the rise in BAT SNA (Fig. 6A,B). During this initial phase (−4 min duration) of the increase in BAT SNA evoked by NMDA nanoinjection into PeF-LH, BAT SNA rose rapidly to a peak level that was 180% greater than that before the NMDA injection (baseline: 263 ± 33% of control vs peak: 736 ± 132% of control, p < 0.05, n = 6) and that was significantly greater (p < 0.05, n = 5, one outlier lacking a distinct early, rapid increase in BAT SNA was excluded) than the level of BAT SNA reached during the same period following Orx-A nanoinjection into rRPa (n = 6). There was no difference (p > 0.05) between the baseline levels of BAT SNA for these two groups.

After the subsidence of the early increase following NMDA nanoinjection into PeF-LH, the increase in BAT SNA was sustained for >1 h and reached a peak increase of 108% (baseline: 263 ± 33% of control vs peak: 547 ± 90% of control, p < 0.05, n = 6), which was not different (p > 0.05) from the sustained increase in BAT SNA following Orx-A nanoinjection into rRPa. The BAT thermogenic response to nanoinjection of NMDA into PeF-LH consisted of an increase in T BAT from 32.7 ± 0.4°C at baseline to a peak of 33.8°C ± 0.5°C (p < 0.05, n = 6), which contributed to the increase in expired CO2 (baseline: 3.7 ± 0.2% vs peak: 4.0 ± 0.2% peak, p < 0.05, n = 6) and in Tcore (36.2 ± 0.2°C baseline vs 36.7 ± 0.2°C peak; p < 0.05; n = 6) (Fig. 6A,B). Activation of neurons in PeF-LH with NMDA also evoked a brief (~2 min) fall in arterial pressure (94 ± 6 mmHg baseline vs 79 ± 7 mmHg nadir, p < 0.05, n = 6) immediately following the NMDA injection in PeF-LH (Fig. 6A,B), which did not occur following Orx-A nanoinjection into rRPa (Fig. 3A,B).
basal level of ongoing BAT SNA suggests that if orexin release in the rRPa is solely responsible for the increases in BAT SNA and BAT thermogenesis, evoked by NMDA nanoinjection into PeF-LH, then, under warm conditions, activation of neurons in PeF-LH should have no effect on BAT thermogenesis. Nanoinjection of NMDA in the PeF-LH (Fig. 7C,D) in rats whose T\text{core} was maintained at a level (37–38°C) that completely silenced spontaneous BAT SNA, did not produce a change in any of the recorded variables (Fig. 7A,B). In 3 of 4 rats, several large bursts of BAT SNA occurred immediately after the injection of NMDA into the PeF-LH (Fig. 7A) however, as was the case following the injection of Orx-A into rRPa in warmed rats, these bursts were also sparse, short-lived and highly variable (Fig. 5A,B).

**Injection of SB-334867 in rRPa**

To establish the role of orexin receptor activation in the parallel increases in BAT SNA and BAT thermogenesis evoked by nanoinjections of Orx-A in the rRPa and of NMDA in the PeF-LH we sought to pretreat the rRPa with a putatively specific antagonist of the Orx-1-R, SB334867 under cool skin and core temperature conditions that evoked a small amount of spontaneous BAT SNA. Surprisingly, nanoinjection of SB334867 (3 nmol) into rRPa (Fig. 8C,D) produced a sustained increase in BAT SNA and BAT thermogenesis (Fig. 8A,B) that was qualitatively similar to that evoked by similar application of Orx-A in rRPa (Fig. 3A,B) or in PaPy (Fig. 4A,B), but of shorter duration than either of the latter responses (compare Fig. 8 with Figs. 3, 6). SB334867 nanoinjection into rRPa evoked a marked and rapid increase in BAT SNA (baseline: 499 ± 136% of control; p < 0.05, n = 5), in T\text{BAT} (baseline: 33.7 ± 0.7°C vs peak: 35.4 ± 0.7°C; p < 0.05; n = 5), in expired CO₂ (baseline: 3.6 ± 0.2% vs peak: 3.9 ± 0.2%, p < 0.05, n = 5) and in HR (baseline: 413 ± 10 bpm vs peak: 442 ± 10 bpm, p < 0.05, n = 5) (Fig. 8A,B). Injection of the vehicle into rRPa was without effect. Even nanoinjection of a lower dose (6 pmol) of SB334867 in rRPa also caused a marked increase of BAT SNA (baseline: 235 ± 76% of control vs peak: 624 ± 60% of control, p < 0.05, n = 3). In addition, nanoinjection of SB334867 (3 nmol) in rRPa following nanoinjection of Orx-A (12 pmol, n = 2) into rRPa or after nanoinjection of NMDA in PeF-LH (12 pmol, n = 2) was without effect (data not shown) on the increased BAT SNA or BAT thermogenesis elicited by Orx-A in rRPa or activation of PeF-LH neurons.

**Discussion**

This study reveals a brainstem mechanism for the potent orexinergic stimulation of energy expenditure and thermogenesis in BAT which could contribute to orexin-mediated thermoregulatory and metabolic changes during a variety of behaviors, including arousal, stress and feeding. Our principal physiological findings are that (1) Orx-A increases BAT SNA and BAT thermogenesis via direct actions in the rRPa and PaPy, sites of sympathetic premotor neurons controlling BAT thermogenesis; (2) activation of neurons in the orexinergic PeF-LH increases BAT SNA and BAT thermogenesis in a manner paralleling that evoked by Orx-A in rRPa; and (3) these evoked increases in BAT thermogenesis require an ongoing, basal level of BAT SNA. These results are consistent with a role for the PeF-LH orexinergic input to rRPa and PaPy as a gain control, amplifying the excitation of BAT sympathetic premotor neurons to enhance BAT thermogenesis. Our delineation of the precise distribution of orexin neurons that project to rRPa and PaPy provides an anatomical basis for the influence of a direct orexinergic input to the rRPa and PaPy on BAT thermogenesis. Indeed, the entire rostrocaudal extent of neurons in rRPa and PaPy infected following PRV injections into BAT were surrounded by a dense network of highly varicose orexin fibers. Further, orexin neurons located in the PeF-LH became infected later than neurons in rRPa and PaPy, consistent with the retrograde labeling of orexin neurons in PeF-LH following CTb injections in rRPa or PaPy.

BAT thermogenesis is controlled by BAT sympathetic premotor neurons in the rRPa and PaPy. Their activation by injection of
GABA<sub>A</sub> antagonists or excitatory amino acid agonists increases BAT SNA and thermogenesis even in animals whose T<sub>Core</sub> is maintained at a level preventing spontaneous BAT SNA (Morrisson et al., 1999; Madden and Morrison, 2003). In the present study, nanoinjection of Orxa-A in the rRPa produced a strong and long-lasting increase in BAT SNA and thermogenesis only when the rats were in a slightly cooled condition that permitted spontaneous BAT SNA, elicited by skin and core cooling, nanoinjection of the orexin antagonist, SB-334867 (dashed line), into the rRPa elicited a marked and sustained (>30 min) enhancement of BAT SNA, T<sub>BAT</sub>, and expired CO<sub>2</sub> (Exp CO<sub>2</sub>), as well as an increase in HR. T<sub>b</sub> was not changed. The means ± SEM (n = 5) of the time courses of the physiological variables (points are 30 s averages of the variable values) during the nanoinjection of SB-334867 into rRPa are shown in B. *p < 0.05, significant increases comparing average variable values during the 1 min window before SB-334867 injection vs 1 min at the peak. C. Histological section showing the microsphere deposit at the SB-334867 nanoinjection site (white arrowhead) in rRPa for the responses illustrated in A. D. The distribution of SB-334867 nanoinjection sites (black dots) are plotted on an atlas drawing (Paxinos and Watson, 2007) containing the rRPa at the level of the facial nucleus (7n). Py, Pyramidal tract.

We observed a dense network of orexin fibers and terminals surrounding PRV-infected neurons in the PaPy after PRV injection into BAT. In addition, Orxa-A/CTb double-labeled neurons following injection of CTb into PaPy had a similar distribution throughout the PeF-LH orexergic field as those retrogradely labeled following CTb injection into rRPa. The temporal sequence of PRV infection (rRPa and PaPy infection preceding infection in PeF-LH) and the similar distribution of PRV-infected neurons in the PeF-LH after PRV injection into BAT and of CTb-labeled neurons in PeF-LH after CTb injection in rRPa and PaPy, provides strong support for an involvement of the direct orexergic projection to the rRPa and the PaPy in regulating BAT thermogenesis. Although putative BAT sympathetic premotor neurons in the PaPy were identified after PRV injection into BAT (Cano et al., 2003; Berthoud et al., 2005) and their role in BAT thermogenesis was proposed (Blessing, 2005), the present results provide the first direct demonstration of a physiological role for these neurons in the control of BAT thermogenesis, in this case, mediated by orexin. Injection of orexin into the PaPy and into the rRPa elicited similar effects on BAT thermogenesis, consistent with both populations of BAT sympathetic premotor neurons receiving a common orexergic input from PeF-LH.

As noted in previous reports (Hermann et al., 1997; Samuels et al., 2004; Yoshida et al., 2009), our retrograde tracer injections into rRPa also labeled a distinct, non-orexergic population of neurons in the dorsal DMH and dorsal hypothalamic area. The excitations of BAT SNA evoked from activation of neurons in the DMH (Cao et al., 2004) or in the ventrolateral LH (Cerri and Morrison, 2005) exhibited a markedly shorter time course than those evoked here by nanoinjection of NMDA into the PeF-LH or by Orxa-A into the rRPa. This observation is consistent with the unique character of the BAT thermogenic response evoked from the PeF-LH region containing orexin neurons, potentially due to a prolonged and potent modulatory effect of orexin on the excitability of BAT sympathetic premotor neurons in rRPa. However, determining whether the amplification of BAT thermogenesis following activation of PeF-LH neurons is mediated by orexin receptors in rRPa must await the availability of improved orexin receptor antagonists.

Although we sought to determine whether the increased BAT thermogenesis evoked by activation of neurons in the PeF-LH was mediated by orexin release in rRPa, we found that nanoinjection of the Orxa-R antagonist, SB 334867, in rRPa had a strong agonist-like effect, stimulating a significant and prolonged increase in BAT SNA and BAT thermogenesis (Fig. 8). Subsequent injection of SB334867 in rRPa had no effect on the amplitudes or time courses of the increases in BAT SNA and BAT thermogenesis evoked by either Orxa-A injection in rRPa or NMDA injection in PeF-LH. Consistent with our observation, SB334867 was reported to have partial agonist activity (Bengtsson et al., 2007) and chronic intracerebroventricular infusion of SB334867 acted similarly to orexin to increase BAT temperature in free-behaving rats (Verty et al., 2010). Although these observations call into question the efficacy of this drug as a selective orexin receptor antagonist, complexities of the microcircuity in rRPa or the localization of orexin receptors to specific neu-
ronal populations could explain the ability of SB334867 to activate BAT thermogenesis.

NMDA stimulation of neurons in the PeF-LH evoked an increase in BAT SNA that included an early, rapidly rising, but transient phase that was not observed in the activation of BAT SNA following Orx-A injection in rRPa (compare Figs. 3, 6). Since a significant fraction of the neurons in PeF-LH that project to rRPa are not Orx-A-ir, it may be that NMDA injection activates both the non-orexinergic population of PeF-LH neurons, resulting in a strong, initial excitation of BAT SNA, as well as the orexinergic neurons in PeF-LH, resulting in the second, long-lasting activation of BAT SNA. Indeed, all the characteristics of the long-lasting response evoked from NMDA activation of PeF-LH neurons closely resemble those elicited by direct microinjection of orexin in rRPa, including the long duration and the requirement for an ongoing level of basal BAT SNA. Another possibility is that this initial phase of BAT SNA excitation may arise from glutamate released from PeF-LH neurons, including ~50% of the orexin neurons, that contain vesicular glutamate transporters (VGLUTs) (Rosin et al., 2003), although whether VGLUT-containing, PeF-LH neurons project to the rRPa is unknown. We could not test the glutamatergic nature of the initial BAT SNA excitation from NMDA injection in the PeF-LH since cold-evoked BAT SNA is blocked by blockade of glutamate receptors in rRPa (Nakamura and Morrison, 2007). Finally, we cannot rule out an indirect pathway, since PeF-LH neurons also project to other brain regions involved in thermoregulation and metabolism, including the arcuate nucleus, median and mediodorsal preoptic areas, and the DMH (Peyron et al., 1998), that could mediate the early, transient activation of BAT SNA.

In conclusion, this study demonstrates a potent and long-lasting modulatory role of orexin, acting in the rRPa and PaPy, to amplify ongoing BAT sympathetic outflow, BAT thermogenesis and BAT energy expenditure. The localization of orexinergic neurons solely in the PeF-LH, the demonstration of retrogradely labeled, orexinergic neurons in PeF-LH following PRV injection into BAT and after CTb injections into rRPa and PaPy, and the finding that the BAT sympathetic and thermogenic responses to PeF-LH activation parallel those to Orx-A injection into rRPa and PaPy provide strong support for the conclusion that orexin release in the rRPa and PaPy from neurons in PeF-LH could play a significant role in setting the overall tone of BAT thermogenesis and energy expenditure and, in turn, body temperature, particularly across sleep–wake cycles, during arousal or periods of increased vigilance requiring activity or in response to dietary influences. These findings, coupled with the increasing evidence supporting an interaction between BAT energy expenditure and obesity (Hamann et al., 1998; Feldmann et al., 2009; Vijgen et al., 2011), dovetail with indications of the influence of the orexinergic system on energy expenditure from physical activity (Novak et al., 2003), to suggest that the orexinergic system is a key determinant of overall energy expenditure and thus, body weight regulation, such that a reduced orexinergic influence could increase the risk of obesity and metabolic syndrome as in narcolepsy (Poli et al., 2009), while augmented orexin activity could contribute to a lean phenotype (Funato et al., 2009).

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