Leptin-sensitive sensory nerves innervate white fat

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Leptin-sensitive sensory nerves innervate white fat. Am J Physiol Endocrinol Metab 304: E1338–E1347, 2013. First published April 23, 2013; doi:10.1152/ajpendo.00021.2013.—Leptin, the primary white adipose tissue (WAT) adipokine, is thought to convey lipid reserve information to the brain via the circulation. Because WAT responds to environmental/internal signals in a fat pad-specific (FPS) manner, systemic signals such as leptin would fail to communicate such distinctive information. Saturation of brain leptin transport systems also would fail to convey increased lipid levels beyond that point. WAT possesses sensory innervation exemplified by proven sensory-associated peptides in nerves within the tissue and by viral sensory nerve-specific transneuronal tract tracer, H129 strain of herpes simplex virus 1 labeling of dorsal root ganglia (DRG) pseudounipolar neurons, spinal cord and central sensory circuits. Leptin as a paracrine factor activating WAT sensory innervation could supply the brain with FPS information. Therefore, we tested for and found the presence of the long form of the leptin receptor (Ob-Rb) on DRG pseudounipolar neurons immunohistochemically labeled after injections of Fluorogold, a retrograde tract tracer, into inguinal WAT (IWAT). Intra-IWAT leptin injections (300 ng) significantly elevated IWAT nerve spike rate within 5 min and persisted for at least 30 min. Intra-IWAT leptin injections also induced significant c-Fos immunoreactivity (ir), indicating neural activation across DRG pseudounipolar sensory neurons labeled with Fluorogold IWAT injections. Intrapitoneal leptin injection did not increase c-Fos-ir in DRG or the arcuate nucleus, nor did it increase arcuate signal transducer and activator of transcription 3 phosphorylation-ir. Collectively, these results strongly suggest that endogenous leptin secreted from white adipocytes functions as a paracrine factor to activate spinal sensory nerves innervating the tissue.

Dorsal root ganglia; tract tracing; electrophysiology; white adipose tissue

White adipose tissue (WAT) mass is a cardinal index of obesity. In animal models, surgical removal of an individual white adipose depot results in a fully compensatory increase in fat mass across the remaining depots, suggesting that WAT mass may be a regulated physiological variable (37). The biological mechanisms responsible for this putative regulation of WAT mass are poorly understood, but recent results provide morphological and functional evidence supporting the physiological significance of neuroendocrine communication between WAT and the central nervous system (CNS) (e.g., for review, see Refs. 6 and 10). Injections of conventional neuronal tract tracers directly into adipose tissue have identified direct postganglionic sympathetic innervation of WAT (65), and subsequent studies using WAT injections of the transneuronal retrograde viral tracer, pseudorabies virus [PRV (3, 54, 55)], have revealed an extensive sympathetic central efferent circuit projecting ultimately to WAT, including labeling of multiple forebrain, midbrain, and hindbrain regions implicated in the control of energy balance (for review, see Ref. 6). Accordingly, surgical transection and chemical blockade of the postganglionic sympathetic innervation of WAT each block the central transneuronal transport of WAT-injected viral tract tracers (23) and prevent lipid mobilization in response to several lipolytic stimuli (e.g., Refs. 8, 14, 34, and 66).

This efferent neuronal pathway linking brain to WAT is driven by a variety of factors, including central actions of the primarily adipose tissue-derived hormone leptin (42, 59, c.f., 44, 47) and by central melanocortin 4 receptor (MC4R) signaling (42). Mediobasal hypothalamic application of leptin suppresses WAT lipogenesis, and this suppression is blocked by surgical denervation and chemical sympathectomy of WAT using 6-hydroxydopamine (12). In addition, MC4R have been localized to multiple central hypothalamic, brainstem, and sympathetic preganglionic neuronal populations projecting to WAT (55), suggesting a role for central neuronal MC4R signaling in the control of adipose tissue function. Consistent with this pattern of expression, central intracerebroventricular administration of the MC3/4 receptor agonist MTII promotes WAT lipolysis (53).

Such data support the idea that neural communication between the brain and WAT is important in the control of adipose tissue function. By contrast, relatively little is known regarding the neurobiological basis for sensory neural communication between WAT and the CNS, beyond the hypothesized role of circulating leptin. In laboratory rats, retrograde tracer application of True Blue into WAT labels pseudounipolar sensory neurons of the dorsal root ganglia [DRG (21)], and results from histological studies of WAT pads in Siberian hamsters have shown substance P- and calcitonin gene-related peptide immunoreactivity (ir), two proven sensory neuropeptides (50, 51). WAT application of the sensory neurotoxin capsaicin, which selectively destroys unmyelinated C- and some thinly myelinated A-delta nerve fibers, recapitulates the compensatory increase in remaining fat pad mass seen after lepimetry, suggesting that a lack of sensory output from WAT to brain is interpreted as tantamount to the removal of the WAT pad, denoting a lipid deficit even though one does not exist (50). Thus, sensory neural communication from WAT afferents may be important in the neural feedback control of adiposity. In Siberian hamsters, injections of anterograde transneuronal viral tract tracer H129 strain of herpes simplex virus 1 (HSV-1) into epididymal (EWAT) and inguinal (IWAT) WAT identify spinal sensory afferents in the DRG, as well as projections to many sites across the neuroaxis that overlap with neuronal populations with WAT sympathetic nervous system (SNS) outflow (56). This pattern of overlap is consistent with the idea...
that neural feedback loops exist between WAT sensory afferents and SNS outflow neurons innervating WAT. Indeed, preliminary studies where both H129 and PRV are injected into the same WAT depot to label sensory input from and sympathetic output to WAT, respectively, reveal dually labeled individual neurons in a variety of brain sites, suggesting a morphological basis for SNS-sensory feedback loops (57). In addition, in anesthetized hamsters, we found that injections of the glucoprivic agent 2-deoxy-d-glucose, which drives sympathetic outflow and lipolysis (11), significantly increases the rate of action potentials in decentralized sensory nerve fibers innervating IWAT. These data suggest the possibility that sensory afferents are responsive to factors secreted by adipose tissue. Indeed, in laboratory rats, injection of the largely adipose tissue-derived cytokine leptin directly into EWAT increases afferent neural activity in the peripheral cut end of the nerves supplying this pad, beginning 30 min after leptin injection (40). This 30-min time point, however, does not address whether the increase in sensory neural activity is mediated by leptin signaling at putative functional leptin receptors expressed in WAT afferents or is secondary to other indirect neuroendocrine consequences of leptin action at peripheral and/or central leptin receptors.

From the perspective of possible WAT sensory-SNS feedback loops, elevations in renal sympathetic nerve outflow have been reported as early as 5 min following leptin injections directly into EWAT (58). It is unknown, however, whether leptin-induced afferent activity in WAT sensory nerves occurs at such early time points, and it remains unclear whether leptin-induced WAT sensory nerve activity is mediated by leptin receptor signaling in WAT afferent neurons. A single previous report noted evidence of the long form of the leptin receptor (Ob-Rb) in laboratory rat DRG as demonstrated by immunohistochemistry (IHC), Western blot, and RT-PCR (16). In the present study, we tested: 1) for the presence Ob-Rb in DRG pseudounipolar neurons by IHC for Ob-Rb-ir in WAT sensory nerves labeled by injections of Fluorogold (FG), 2) whether intra-WAT leptin injections trigger increased acute sensory nerve electrophysiological activity, and 3) whether intra-WAT injection of leptin induced c-Fos-ir, an accepted index of neural activation (25), in DRG pseudounipolar sensory neurons. Collectively, we hypothesize that locally released leptin functions in a paracrine manner to activate WAT afferents and thereby signal the brain of changes in lipid storage or metabolism in white adipocytes.

METHODS

All procedures were approved by the Georgia State University and Albert Einstein College of Medicine Institutional Animal Care and Use Committees and are in accordance with Public Health Service and United States Department of Agriculture guidelines.

Animals

Male Siberian hamsters (Phodopus sungorus; ~3–4 mo old) were single-housed after being selected from our breeding colony (16:8-h light-dark cycle; lights on at 0200). Room temperature was maintained at 21 ± 2°C, with relative humidity at ~50 ± 10%. Hamsters were single-housed for 7–10 days before any treatments.

IHC for Signal Transducer and Activator of Transcription 3 Phosphorylation, c-Fos, and Ob-Rb

Free-floating brain sections (30 μm thickness) were subsequently rinsed in PBS (3 × 10 min), in 0.3% glycine in PBS (25 min), in PBS (3 × 5 min), in 0.03% sodium dodecyl sulfate in PBS (25 min), and in PBS (3 × 5 min) followed by a 30-min incubation in 10% normal goat serum (NGS) in PBTx (PBS containing 0.3% Triton X-100). Sections were then incubated in primary antibody for rabbit anti-signal transducer and activator of transcription 3 (STAT3) phosphorylation (pSTAT3) (1:1,000; no. 9145; Cell Signaling Technology, Danvers, MA) or rabbit anti-c-Fos (1:800; sc-52; Santa Cruz Biotech, Santa Cruz, CA) with 2% NGS in PBTx with 0.3% sodium azide for 2 days. Next, slices were rinsed in PBS (3 × 5 min), incubated for 2 h in goat anti-rabbit secondary antibody (1:200; Vector Laboratories, Burlingame, CA) in 2% NGS in PBTx, rinsed in PBS (3 × 5 min), and incubated for 1 h in Alexa 594-conjugated streptavidin (1:1,000; Molecular Probes, Eugene, OR). For immunohistochemical controls, the primary antibody was either omitted or preadsorbed with the immunizing peptide overnight. All steps were performed at room temperature. Sections were mounted onto slides (Superfrost Plus; VWR International, West Chester, PA) and cover slipped using ProLong Gold Antifade Reagent (Molecular Probes).

DRG were sliced longitudinally into 20-μm serial sections and mounted in three series onto slides with every forth section on the same slide. After drying on slides, the sections were rehydrated and processed for detection of c-Fos antigen (1:200 dilution) and Ob-Rb antigen (1:200; Neuromics, Edina, MN) as described above, except that the incubation period was for 3 days for the primary and 1 day for the secondary antisera.

Experiment 1: Do DRG Innervating WAT Possess Ob-Rbs?

The purpose of this experiment was to test whether DRG innervating WAT express Ob-Rbs. Previously, Ob-Rb immunohistochemical localization has been questioned due to inconsistencies with studies using IHC methodology usually based on the lack of specificity of Ob-Rb antibodies (e.g., Ref. 20). A relatively new antibody (chicken anti-rat Ob-Rb; Neuromics), however, has been repeatedly characterized for specificity (4, 17, 26, 38), including additional verification via Western blot (38). We additionally tested for specificity by preadsorbing the antibody with a recombinant Ob-Rb peptide. The adsorption control resulted in no Ob-Rb-ir. Therefore, we first labeled the DRG innervating IWAT using the tract tracer FG (Fluorochrome, Denver, CO) and then processed the tissue for Ob-Rb-ir, both described below. Thus, we sought double-labeled DRG pseudounipolar neurons positive for FG and Ob-Rb-ir.

FG labeling of IWAT sensory innervation of DRG. Adult male hamsters (n = 5) were single housed for 1 wk. The animals were then anesthetized with 2% isoflurane; the back haunch area over the IWAT pad was shaved and wiped with a 10% povidone-iodine solution (Betadine); and an incision was made around the IWAT pad area to expose it, being careful not to damage the underlying neural and vascular provisions to the area. A 2% FG solution was injected at eight sites across the IWAT pad. At each site, the injection needle was inserted ~4 mm, 1 μl of FG was injected, and the needle was held in place for ~1 min to minimize reflux up the outside of the needle (for a total of 8 μl for 8 loci). Care was taken to keep the IWAT pad moist during the procedure. The incision was closed with sterile wound clips, and nitrofurazone antimicrobial powder was applied to prevent infection. Two weeks post-FG injection, hamsters were perfused for histological analyses as described below.

Harvesting of DRG and histology. Histological preparation for this experiment and for experiment 3 follows in brief. Hamsters were overdosed with pentobarbital sodium (300 mg/kg ip) and perfused transcardially with heparinized (0.02%) saline and paraformaldehyde (4%) in 0.1 M phosphate buffer (pH 7.4). DRG associated with spinal cord vertebrae T11–L3 were harvested and then carefully removed, postfixed in the same fixative for 10 min, and then transferred to a sucrose solution (30%) with 0.1% sodium azide and stored at 4°C until they were sectioned on a freezing stage sliding microtome at 20
µm. Immediately after being sliced, the DRG were thaw-mounted onto slides and stored at 4°C until processing for IHC.

Histological quantification analysis. Quantification of labeling for this experiment and for experiment 3 follows in brief. DRG sections were viewed and captured under ×200 magnification with an Olympus BX41 fluorescence microscope (Olympus, Tokyo, Japan) equipped with a digital camera (DP70; Olympus) and appropriate filters for FG (excitation wavelength 360–370 nm) and Alexa 594 (excitation wavelength 560–596 nm). Quantification of c-Fos-ir, FG, and Ob-Rb-ir was performed on every third section to eliminate the likelihood of counting neurons two times. Images were acquired using Adobe Photoshop CS5 (Adobe Systems, San Jose, CA) and adjusted only for brightness, contrast, and sharpness. c-Fos-ir was considered positive based on the appropriate size and shape of nuclei. For FG counts, faint FG-labeled neurons were excluded from the counting. The number of c-Fos-ir nuclei within FG-positive neurons was represented as a percentage from the total FG-labeled neurons.

**Experiment 2: Does Intra-WAT Leptin Increase Ob-Rb Activation in DRG Innervating WAT?**

The purpose of this experiment was to test whether direct injection of leptin into WAT stimulates DRG pseudounipolar neurons innervating WAT, as initially suggested by Niijima (40, 41) through his intra-EWAT injections and electrophysiological measures of sensory nerve activity. Here we gave intra-IWAT injection of leptin in hamsters that first had the tracer FG injected into IWAT to label the DRG pseudounipolar afferent neurons. Two weeks later, they were injected with leptin intra-WAT to test for activation of the labeled neurons, as indicated by c-Fos-ir, an accepted marker of neural activation (25). Thus, we sought double-labeled DRG pseudounipolar neurons positive for FG and c-Fos-ir.

**FG sensory nerve tract tracing.** Adult male hamsters (n = 12) were single housed for 1 wk. The animals were then anesthetized with 2% isoflurane; the back haunch area over the IWAT pad was shaved and wiped with a 10% povidone-iodine solution (Betadine); and an incision was made around the IWAT pad area to expose it, being careful not to damage the underlying neural and vascular provisions to the area. FG injections were performed in the same manner as described above in FG labeling of IWAT sensory innervation of DRG. Seven days post-FG injections, the hamsters were handled for 5–10 min every day to adapt them to the handling/anesthesia administration associated with the intra-IWAT leptin injections. After handling on the 7th day, all hamsters were food deprived for 48 h to inhibit endogenous leptin secretion (1, 9, 24). At the end of the 48-h period, the animals were anesthetized with ketamine (100 mg/kg)/xylazine (10 mg/kg), the area over IWAT was shaved and, with a 10% povidone-iodine solution (Betadine); and an incision was made around the IWAT pad area to expose it, being careful not to damage the underlying neural and vascular provisions to the area. FG injections were performed in the same manner as described above in FG labeling of IWAT sensory innervation of DRG. Seven days post-FG injections, the hamsters were handled for 5–10 min every day to adapt them to the handling/anesthesia administration associated with the intra-IWAT leptin injections. After handling on the 7th day, all hamsters were food deprived for 48 h to inhibit endogenous leptin secretion (1, 9, 24). At the end of the 48-h period, the animals were anesthetized with ketamine (100 mg/kg)/xylazine (10 mg/kg), the area over IWAT was shaved, and with a 10% povidone-iodine solution was applied. IWAT was exposed via an incision just lateral to the fat pad. Hamsters were injected intra-IWAT with leptin (murine recombinant; Sigma Chemical, St. Louis, MO; see directly below for details) and kept under anesthesia for 1 h, at which time they were perfused for histological analyses. Single (FG or c-Fos-ir)- and double (FG + c-Fos-ir)-labeled DRG pseudounipolar neurons were quantified microscopically by eye as detailed above in experiment 1.

**Intra-WAT leptin injection.** Long day-housed Siberian hamsters, such as those used here, are relatively leptin resistant, as indicated by a lack of body and fat mass decrease to exogenously administered leptin compared with a robust decrease in their short day-housed counterparts (31, 48) and associated with a marked increased arcuate nucleus (Arc) suppressor of cytokine signaling-3 gene expression (60), a negative regulator of Ob-Rb associated with a marked increased arcuate nucleus (Arc) suppressor of cytokine signaling-3 gene expression (60), a negative regulator of Ob-Rb associated with a marked increased arcuate nucleus (Arc) suppressor of cytokine signaling-3 gene expression (60), a negative regulator of Ob-Rb associated with a marked increased arcuate nucleus (Arc) suppressor of cytokine signaling-3 gene expression (60), a negative regulator of Ob-Rb associated with a marked increased arcuate nucleus (Arc) suppressor of cytokine signaling-3 gene expression (60), a negative regulator of Ob-Rb associated with a marked increased arcuate nucleus (Arc) suppressor of cytokine signaling-3 gene expression (60), a negative regulator of Ob-Rb associated with a marked increased arcuate nucleus (Arc) suppressor of cytokine signaling-3 gene expression (60), a negative regulator of Ob-Rb associated with a marked increased arcuate nucleus (Arc) suppressor of cytokine signaling-3 gene expression (60), a negative regulator of Ob-Rb associated with a marked increased arcuate nucleus (Arc) suppressor of cytokine signaling-3 gene expression (60), a negative regulator of Ob-Rb associated with a marked increased arcuate nucleus (Arc) suppressor of cytokine signaling-3 gene expression (60), a negative regulator of Ob-Rb associated with a marked increased arcuate nucleus (Arc) suppressor of cytokine signaling-3 gene expression (60), a negative regulator of Ob-Rb associated with a marked increased arcuate nucleus (Arc) suppressor of cytokine signaling-3 gene expression (60), a negative regulator of Ob-Rb associated with a marked increased arcuate nucleus (Arc) suppressor of cytokine signaling-3 gene expression (60), a negative regulator of Ob-Rb associated with a marked increase...
A surgical plane of anesthesia was maintained by 0.1-ml intravenous injections of ketamine/xylazine as necessary to maintain areflexia to toe pinch. Heart rate and blood oxygenation were monitored throughout all experiments by a tail pulse oximeter (Nonin Medical, Plymouth, MI). A 2-cm incision was made along the ventrum to expose the IWAT pad. A 1-cm segment of the inguinal nerve was exposed by isolating the IWAT pad from the surrounding tissue. Teflon tape was placed under the neurovascular bundle, and warm mineral oil was then added to aid in dissecting the epineurium from the nerve fibers and blood vessel. With the use of a dissecting microscope (×30–40), a strand from the neurofiber bundle was teased apart and severed to create a decentralized strand leaving as much of the trunk in situ as possible. The lateral edges of the exposed pad were packed with gelfoam to minimize bleeding and form a well that held a small pool of warmed (37°C) mineral oil over the recording sites along the nerve penetrating IWAT. Three individual 30-gauge needles spaced 0.7 cm apart were inserted directly into IWAT spanning the medial ventral to lateral dorsal plane of the pad. Injectors were attached via PE-10 tubing to CMA microdialysis pumps driving 10-μl Hamilton syringes with either saline vehicle or recombinant murine leptin (Peptotech, Rocky Hill, NJ). An air bubble, placed in each infusion line, separated the saline baseline infusion from the subsequent leptin or saline vehicle infusion. Three hundred nanograms total of leptin were administered in three simultaneous semibolus injections of 100 ng/5 μl saline each minute, starting at 300 ng intraperitoneal leptin or saline vehicle as a control if all the intra-IWAT leptin leaked into the peritoneum. At the end of neurophysiological studies, animals were killed by overdose with concentrated pentobarbital sodium (300 mg/kg ip).

Neurophysiological data analysis. Afferent discharges were fed to a Grass P511 AC preamplifier and monitored on line with a Haer audio monitor and Tektronix digital and analog oscilloscopes. In addition, amplified signals are fed to a dual time-amplitude window discriminator (BAK, DDIS-1) to determine the number of spikes occurring above basal voltage to isolate single units from the signal, with surgical and/or stereotactic isolation leaves more than one unit in the signal. Isolated units are triggered to appear on a second digital oscilloscope (Tektronix 2211) following an interposed delay (BAK AD-1, 0.1–3 ms delay range) to confirm that each isolated unit produces a reliable and unique time-amplitude signature. All isolated units above baseline were also recorded in real time using PowerLab Chart (version 5; AD Instruments, Colorado Springs, CO) and subsequently analyzed using the PowerLab spike histogram module (version 5.3; AD Instruments). A digital oscilloscope record was simultaneously written to disk for 50 consecutive spontaneously occurring spikes to confirm the consistency of the isolated units above baseline. A significant multiunit response to a leptin stimulus was defined as a change in the number of total discharges in the 30- to 60-s poststimulus interval of at least 1.5 standard deviations from the average number occurring in 30- to 60-s bins of spontaneous prestimulus activity. These criteria are consistent with previous studies that have successfully recorded and analyzed peripheral IWAT adipose tissue afferent neurophysiological responses (56).

Statistical Analyses

The electrophysiological recordings were analyzed statistically using an ANOVA for repeated measures (Prism, Graph Pad, GPM4.0). DRG data were analyzed by one-way repeated-measures ANOVA followed by the post hoc Bonferroni’s least-significant difference using Sigma Stat 3.5 software (Systat Software, Chicago, IL). Differences between means were considered significant at P < 0.05. Exact probabilities and test values were omitted for simplicity and clarity of presentation.

RESULTS

Experiment 1: Do DRG Innervating WAT Possess Ob-Rb mRNA?

FG unilaterally labeled the DRG pseudounipolar neurons innervating IWAT associated with spinal cord vertebrae T11–L3 (Fig. 1). A representative photomicrograph of a DRG at the vertebral level L2 shows FG-positive DRG pseudounipolar neurons (Fig. 1A), neurons positive for Ob-Rb-ir (Fig. 1B), and double-labeled neurons that are FG + Ob-Rb-ir positive (Fig. 1, inset). The distribution of DRG pseudounipolar neurons positive for Ob-Rb-ir was relatively evenly distributed across DRG associated with spinal vertebrae segments T13–L3 and were significantly greater than for DRG associated with T11 and T12 (P values < 0.05; Fig. 1B). The electrophysiological recordings were analyzed statistically using the post hoc Bonferroni’s least-significant difference using Sigma Stat 3.5 software (Systat Software, Chicago, IL). Differences between means were considered significant at P < 0.05. Exact probabilities and test values were omitted for simplicity and clarity of presentation.

Control injections of the same dose of leptin (300 ng) injected intra-IWAT instead of intraperitoneally resulted in c-Fos-ir in the Arc (Fig. 2, A and B, top) equivalent to that of the saline vehicle. As a second control for possible leakage from the intra-IWAT injection site into the periphery, we also tested for Arc pSTAT3-ir and found that neither intraperitoneal leptin (300 ng) nor intraperitoneal saline generated significant Arc pSTAT3-ir 30 min later, and did not differ from one another (Fig. 2, C and D, middle). As a positive control for pSTAT3-ir, we injected 10× (3 μg) and 100× (30 μg) the intra-IWAT leptin dose (300 ng) intraperitoneally and found marked and anticipated increases in Arc pSTAT3-ir (Fig. 2, E and F, bottom). Intra-IWAT leptin injections (300 ng) increased c-Fos-ir in DRG relative to saline injections and, more importantly, did so in DRG pseudounipolar neurons that innervated IWAT, as demonstrated by c-Fos-ir in FG-positive neurons (Fig. 3, D–F, bottom), whereas there were fewer c-Fos-ir + FG neurons after intra-IWAT saline injection (Fig. 3, A–C, top). The percentage of FG-labeled DRG pseudounipolar neurons showing c-Fos-ir 1 h after intra-IWAT injection of leptin was significantly greater only at the DRG level associated with L1 (P < 0.05; Fig. 4); however, significance was approached at DRG level T13 (P = 0.08; Fig. 4). More importantly, collapsing across T11 to L3, the percentage of FG-labeled DRG pseudounipolar neurons also exhibiting c-Fos-ir after intra-IWAT leptin injection was significantly greater than compared with that following saline vehicle injection (P < 0.05; Fig. 4, inset).

Experiment 3: Does Intra-WAT Leptin Increase Electrophysiological Activity of Sensory Afferents Innervating WAT?

Baseline activity arising from isolated fibers from the right IWAT ranged from 12 to 22 spikes/30-s bin. Simultaneous injections of saline vehicle at three sites along the dorsal-ventral extent of the right IWAT produced no significant change in the rate of spike activity above baseline levels at any time point tested. By contrast, intra-IWAT injection of a total dose of 300 ng of recombinant rat leptin along these sites significantly elevated IWAT nerve spike rate, beginning within the first 5 min following leptin administration. This elevated rate persisted for the entire 30-min test period, with peak

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activity occurring during the 10-min bin after leptin injection (Fig. 5A).

In separate studies, intraperitoneal injection of 300 μg of leptin (n = 5) or saline vehicle (n = 5) produced no significant response above basal spontaneous activity at any time point (Fig. 5B).

DISCUSSION

Collectively, the results of the present study strongly infer that endogenously secreted leptin from white adipocytes functions as a paracrine factor to activate WAT spinal sensory nerves. We report for the first time the presence of Ob-Rbs in DRG pseudounipolar neurons innervating WAT, as evidenced by the colocalization of Ob-Rb-ir with sensory afferents using IHC and FG. In addition, we also report for the first time the ability of intra-WAT injections of leptin to activate DRG pseudounipolar neurons innervating WAT, as evidenced by colocalization of c-Fos-ir with sensory nerve afferents labeled after FG injections into IWAT. Finally, we extend the finding that intra-WAT leptin injections increase multiunit activity of the sensory nerves innervating EWAT in laboratory rats to similar injection of leptin into IWAT of Siberian hamsters, the latter assessed from decentralized nerve fibers innervating the tissue. Together, these data suggest that, in addition to the leptin conveying to the brain and/or other tissues of a potential adiposity-related signal via the more conventionally accepted circulatory route (49), leptin also may convey this type of information or other information to the brain via a neural route, and do so in a fat pad-specific (FPS) manner rather than doing so in a more global fashion.

Although leptin has been traditionally viewed as a circulating factor that may inform the brain of adiposity levels (64), and although circulating leptin can correlate well with overall adiposity in human/nonhuman animals (e.g., see Refs. 22, 29, and 35), its accuracy in doing so can also be questioned. For example, acutely, circulating leptin rapidly decreases in cold-exposed rats (24) and humans (46) without measurable decreases in adiposity. More “chronically,” although circulating leptin concentrations in humans and rodents are correlated with the degree of adiposity more generally, a considerable part of
the between-individual variance in plasma leptin concentrations is unrelated to the degree of adiposity in humans (for review, see Ref. 28). Thus, circulating leptin can lack fidelity with adiposity. In addition, circulating leptin provides no FPS information to the brain regarding depot lipid levels because, to our knowledge, the same leptin molecule is released from each WAT pad. The ability to transmit FPS information to the CNS seems critical in that FPS increases and decreases in lipid

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**Fig. 2.** Test for diffusion of the 300-ng intra-IWAT leptin injection by injecting this dose ip. Microphotographs taken at the level of the arcuate nucleus. c-Fos-ir 1 h after saline (A) and 300 ng ip leptin (B) injection. Note no difference in c-Fos-ir between saline (A) and 300 ng leptin (B) treatment. Signal transducer and activator of transcription 3 phosphorylation (pSTAT3)-ir 30 min after saline (C), 300 ng leptin (D), 3,000 ng leptin (E), and 30,000 ng leptin (F). Note no difference in pSTAT3-ir between ip injection of saline (C) and 300 ng leptin (D). Bottom, 3-μg (E) and 30-μg (F) leptin injections are positive controls for the ability to detect leptin-induced increases in pSTAT3-ir. Arc, Arcuate nucleus; ME, median eminence; VMH, ventromedial hypothalamic area; 3V, third ventricle. Scale bar = 50 μm.

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**Fig. 3.** Leptin-induced activation of DRG neurons innervating IWAT at the vertebral level T13. A and D: FG-labeled DRG neurons innervating IWAT. B and E: c-Fos-ir 1 h after intra-IWAT injection of saline (A–C) or leptin (300 ng, D–F). C and F: merged images showing c-Fos-ir + FG-labeled neurons (white arrows). Scale bars = 50 μm.
deposition/mobilization are the norm and at least some WAT depots appear to have specific functions in addition to the shared function across pads as a repository for lipid fuels. For example, lipid compensation patterns in WAT depots following partial WAT lipectomy are dependent upon which WAT pads are excised and are not due to the size of the lipid deficit; if the latter were true, this would suggest a general factor predominates, but it does not appear to be so (for review, see Ref. 37). Therefore, it is difficult to reconcile FPS effects with any circulating factor produced by white adipocytes, especially those requiring involvement of the CNS (e.g., lipolysis; for review, see Refs. 5 and 6). Neural communication from WAT to brain, however, could provide the CNS with FPS information. In other organs, parasympathetic nervous system (PSNS) vagal afferents provide the brain with tissue-specific information such as from the stomach, intestines, lung, and pancreas (for review, see Refs. 7, 33, and 43). WAT, however, has no or insignificant PSNS innervation (23), despite an unconfirmed claim to the contrary (32).

Alternatively, spinal sensory nerves could be a unique conduit of FPS information to brain delivered in a rapid manner. Traditionally, sensory information relayed through the ascending spinthalamic and spinocerebellar circuits is considered a pathway to pain and proprioceptive information, respectively (63); however, sensory afferent soma reside in the DRG of the thoracic and more rostral lumbar spinal levels (15) and contain nonnociceptive (i.e., nonpain) visceral receptors involved in the reflexive control or homeostasis (15). Indeed, we labeled these somata in our viral transneuronal tract tracing of sensory outflow from WAT to the brain using the H129 strain of HSV-1 (56). The experiments here further suggest the involvement of spinal sensory afferents innervating WAT that are sensitive to leptin administered locally into the IWAT depot and, by inference, perhaps suggest a paracrine role of leptin in signaling to the CNS lipid-related information.

Previously, Ob-Rbs have been demonstrated in rat DRG by IHC, Western blot, and in situ hybridization (16), but no

**Fig. 4.** Percentage of FG + c-Fos-ir colocalized neurons across the T11–L3 DRG innervating IWAT following intra-IWAT saline (open bars) or 300 ng leptin (filled bars) injections. Total percentage of c-Fos-ir + FG colocalized neurons in collapsed T11–L3 DRG is shown in the inset. *P < 0.05 vs. saline.

**Fig. 5.** A: mean ± SE spike activity/30 s in decentralized afferent nerve fibers arising from the right IWAT in Siberian hamsters immediately before (baseline) and at 5, 10, 20, and 30 min after IWAT injections of a total of 300 ng recombinant rat leptin or saline vehicle injections; n = 6 and 7/group, respectively. *P < 0.05 vs. leptin baseline. #P < 0.05 vs. leptin at all other time points. Inset, trace of neurophysiological response to IWAT leptin in the decentralized WAT nerve (top) and individual spike from the IWAT neurophysiological trace (bottom). Arrow indicates leptin injection. B: mean ± SE spike activity/30 s in decentralized afferent nerve fibers arising from the right IWAT after ip injections of 300 ng recombinant rat leptin or saline vehicle injections (n = 5/group).
attempt was made to establish colocalization with sensory nerves from any individual tissue source, including WAT. Here with a relatively new and specific Ob-Rb antibody, it was possible to identify leptin receptors on DRG pseudounipolar neurons innervating WAT.

From a functional histological standpoint, we found FG-labeled DRG pseudounipolar neurons innervating IWAT are significantly more activated by intra-IWAT leptin injection, as indicated by c-Fos-ir across the DRG associated with vertebral T11–L3 than after saline vehicle injection. This increase in c-Fos-ir by intra-IWAT leptin only was significant at the individual DRG level for the DRG associated with vertebral cord level L1, and nearly at the T13 level ($P < 0.08$), a finding in accordance with the spinal ilioinguinal nerve that originates in L1 and innervates the abdomen and groin area in humans (30). Thus, this appears to be a more general DRG neuronal activation than one that is specific for a single DRG and is consistent with the general trait of sensory afferents from peripheral organs [e.g., liver (61)].

It should be noted that we conducted several tests to help exclude the possibility that the observed effects of intra-IWAT leptin injections were due to diffusion of the cytokine to surrounding tissues or into the intraperitoneal cavity for both the induction of c-Fos-ir in FG-labeled DRG pseudounipolar neurons innervating IWAT as well as for the increases in multiunit neuronal electrophysiological activity (discussed below). Here we injected the same dose/volume of leptin intraperitoneally and assayed c-Fos-ir in the Arc and in the DRG (data not shown) and also assayed pSTAT3-ir in the Arc (Fig. 2). Intraperitoneal leptin (300 ng) did not increase c-Fos-ir or pSTAT3-ir in any site assayed compared with vehicle injections, whereas leptin doses 10 and 100 times higher (3 or 30 µg) clearly increased Arc pSTAT3, demonstrating that the lack of a significantly increased level of leptin-induced Arc pSTAT3 at the 300-ng leptin dose given intraperitoneally was not due to our inability to assay this transcription factor. Collectively, diffusion from the IWAT site of leptin injection does not account for the increased c-Fos-ir in the DRG pseudounipolar neurons innervating IWAT.

The ability of intra-WAT leptin to increase WAT afferent nerve activity was demonstrated previously by Niijima (40) who injected leptin into the EWAT pad of laboratory rats triggering increases in sensory nerve multiunit electrophysiological activity. This finding was ignored by the field, and by us, likely because it was only four years after the discovery of leptin, and the zeitgeist at the time (and persisting today) is that leptin secreted from WAT informs the brain of adiposity levels through the circulation. Those data by Niijima (41) demonstrate an apparent “reflex arc” whereby intra-EWAT leptin injection triggers an increase in sympathetic nerve electrophysiological activity to WAT. This reflex response is hypothesized to be a counterregulatory response to increases in adiposity that might be triggered naturally with increasing WAT lipid levels, thereby increasing SNS-induced lipolysis to keep WAT lipid levels in check (40, 41). Others have expanded these studies to show that intraperigonalad, inguinal, and retroperitoneal WAT leptin injection increases renal sympathetic nerve electrophysiological activity, suggesting, perhaps, a more general activation of SNS activity (52, 58). Furthermore, it recently has been speculated that this possible paracrine effect of leptin on WAT afferent/afferents involved in the reflex arc may malfunction in obesity, promoting, therefore, increases in adiposity (52). The finding here that leptin injections into IWAT increase afferent nerve activity extends the findings of Niijima to another species and occurs more rapidly (within 5 min of injection) rather than the delayed (30 min) increase in afferent neural activity seen by Niijima (40). The rapidity of the electrophysiological response to locally administered leptin in the present study therefore makes it more difficult to attribute the increase in afferent nerve activity to a secondary/indirect neuroendocrine consequence of leptin acting peripherally and/or centrally and demonstrates the possibility of rapid information transit from WAT to the brain. Coupled with the inability of this dose of leptin to trigger the increase in multiunit spike activity when injected intraperitoneally or when a third of the effective dose was injected intra-IWAT, this suggests a ligand-mediated response to leptin via WAT Ob-Rb in pseudounipolar DRG neurons innervating white fat. Thus, the present rapid increase in sensory neural activity induced by intra-IWAT leptin administration suggests a direct effect of leptin on the apparent Ob-Rbs possessed by these sensory neurons, the latter indicated by Ob-Rb-ir in FG-labeled DRG neurons innervating IWAT. The underlying mechanism for the persisting electrophysiological response to the leptin injection here (30 min when the test was stopped), and that of Niijima (40), which lasted for more than 1 h, is unknown.

The obvious and perplexing question is “what information does the presumed local release of leptin from white adipocytes convey to these afferents and what is the response to this information by the CNS?” As noted above, this could be a means of supplying the brain with FPS information on lipid levels for individual WAT pads that could consequently affect the SNS drive to WAT to offset surfeits of stored lipid fuels, as the reflex arc data would suggest (40, 41), and maybe, as also noted above, this reflex arc malfunctions with obesity (52), perhaps due to leptin resistance peripherally at the level of the DRG. This latter notion is a topic for future study. This likely is not the only information sensed by the afferents innervating WAT in these nerve bundles just as afferent nerve bundles from the skin carry sensations of pain, vibration, and pressure. Indeed, we previously found suggestive evidence to indicate that, with increases in the SNS drive to IWAT, induced by glucoprivation (systemic 2-deoxy-D-glucose) known to increase the SNS drive to IWAT (11), sensory nerve multiunit activity increases (56). Those data suggest a factor associated with lipolysis also can increase WAT sensory nerve activity.

In summary, the presence of Ob-Rb mRNA on DRG primary afferent neurons innervating WAT, in combination with the ability to activate these sensory fibers with intra-IWAT injection of leptin that triggers increases in WAT afferent nerve activity and c-Fos-ir in the DRG neurons, suggests locally released leptin by adipocytes could act as a paracrine factor, perhaps conveying lipid levels in a FPS manner to the CNS.

**Perspectives**

In addition to their identification and early characterization of a structural and functional basis for paracrine leptin signaling in WAT via DRG afferents, the present data expose three larger conceptual concerns regarding leptin action. First, these data underscore the importance of evaluating evidence for and the role of peripheral leptin signaling, with particular attention...
focused on paracrine vs. more canonical hormonal/endocrine modes of action. Gastric leptin acting via vagal afferents (e.g., Refs. 2, 13, and 45) provides a significant example supporting the local paracrine action of gastric leptin as a sensory neural stimulus. Second, the identification and characterization of paracrine leptin action at the level of peripheral afferents will naturally promote the search for and comparisons of the intracellular molecular mechanisms responsible for leptin resistance in both peripheral and central neurons. Finally, the identification of paracrine leptin signaling in WAT afferents demands a characterization of how such leptin action changes as a function of the reciprocal metabolic contexts of nutrient deficiency and nutrient excess. Again, recent examples form vagal afferent physiology under different metabolic contexts are instructive, where de Lartigue and colleagues (18) have demonstrated that diet-induced obesity promotes leptin resistance within nodose ganglion neurons of the afferent vagus, via an attenuated pSTAT3 response to leptin. In fact, leptin resistance in these nodose ganglion neurons in diet-induced obese rats preceded its appearance in Arc neurons, demonstrating that the spatiotemporal pattern of leptin resistance may proceed from peripheral to central levels of the nervous system in the metabolic context of nutrient excess.

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DISCLOSURES
The authors have nothing to disclose.

AUTHOR CONTRIBUTIONS

REFERENCES


