White adipose tissue lacks significant vagal innervation and immunohistochemical evidence of parasympathetic innervation

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Giordano, Antonio, C. Kay Song, Robert R. Bowers, J. Christopher Ehlen, Andrea Frontini, Saverio Cinti, and Timothy J. Bartness. White adipose tissue lacks significant vagal innervation and immunohistochemical evidence of parasympathetic innervation. Am J Physiol Regul Integr Comp Physiol 291: R1243–R1255, 2006. First published June 29, 2006; doi:10.1152/ajpregu.00679.2005.—Converging evidence indicates that white adipose tissue (WAT) is innervated by the sympathetic nervous system (SNS) based on immunohistochemical labeling of a SNS marker (tyrosine hydroxylase [TH]), tract tracing of WAT sympathetic postganglionic innervation, pseudorabies virus (PRV) transneuronal labeling of WAT SNS outflow neurons, and functional evidence from denervation studies. Recently, WAT para-SNS (PSNS) innervation was suggested because local surgical WAT sympatheticectomy (sparking hypothesized parasympathetic innervation) followed by PRV injection yielded infected cells in the vagal dorsomotor nucleus (DMV), a traditionally-recognized PSNS brain stem site. In addition, local surgical PRSNS WAT denervation triggered WAT catabolic responses. We tested histologically whether WAT was parasympathetically innervated by searching for PSNS markers in rat, and normal (C57BL) and obese (ob/ob) mouse WAT. Vascular acetylcholine transporter, vasoactive intestinal peptide and neuronal nitric oxide synthase immunoreactivities were absent in WAT pads (retroperitoneal, epididymal, inguinal subcutaneous) from all animals. Nearly all nerves innervating WAT vasculature and parenchyma that were labeled with protein gene product 9.5 (PGP9.5; pan-nerve marker) also contained TH, attesting to pervasive SNS innervation. When Siberian hamster inguinal WAT was sympathetically denervated via local injections of catecholaminergic toxin 6-hydroxydopamine (sparking putative parasympathetic nerves), subsequent PRV injection resulted in no central nervous system (CNS) or sympathetic chain infections suggesting no PSNS innervation. By contrast, vehicle-injected WAT subsequently inoculated with PRV had typical CNS/sympathetic chain viral infection patterns. Collectively, these data indicate no parasympathetic nerve markers in WAT of several species, with sparse DMV innervation and question the claim of PSNS WAT innervation as well as its functional significance.

IN MAMMALS, WHITE ADIPOSE TISSUE (WAT) IS DISTRIBUTED INTO MULTIPLE SUBCUTANEOUS AND VISCERAL DEPOTS (13). THE SIGNATURE CELL TYPE FOUND IN WAT IS THE WHITE ADIPOCYTE WITH ITS CHARACTERISTIC LARGE SINGLE TRACYLGLYCEROL-CONTAINING LIPID DROPLET. UNDER CONDITIONS OF PROLONGED FASTING, EXERCISE, STRESS, OR COLD EXPOSURE, RAPID AND PRECISE NEUROHUMORAL MECHANISMS INDUCE WHITE ADIPOCYTE LIPOLYSIS THROUGH THE CATABOLISM OF TRACYLGLYCEROL INTO FREE FATTY ACIDS AND GLYCEROL THAT ARE SUBSEQUENTLY RELEASED INTO THE CIRCULATION (FOR REVIEW, SEE REF. 34). IN ADDITION TO THIS FUNDAMENTAL ROLE IN STORING AND MOBILIZING LIPID IN RESPONSE TO ENERGY NEEDS, WAT ALSO IS REGARDED AS AN ENDOCRINE ORGAN (FOR REVIEW, SEE REF. 38). INDEED, WHITE ADIPOCYTES PRODUCE AND SECRETE SEVERAL BIOACTIVE MEDIATORS (COLLECTIVELY NAMED ADIPOKINES) THAT INFLUENCE ENERGY HOMEOSTASIS, GLUCOSE AND LIPID METABOLISM, COAGULATION AND FIBRINOLYSIS, IMMUNE RESPONSES, AND REPRODUCTION (38, 39). WAT IS INNervATED BY THE SYMPATHETIC NERVOUS SYSTEM (SNS), WHICH IS THE PRINCIPAL INITIATOR OF WAT LIPID MOBILIZATION (FOR REVIEWS, SEE RefS. 7 AND 8). FURTHERMORE, NOREPINEPHRINE (NE), THE PRIMARY SNS POSTGANGLIONIC NEUROTENSOR, INHIBITS INSULIN-INDUCED LEPTIN SECRETION FROM DIFFERENTIATED WHITE FAT CELLS IN CULTURE (10), THUS SUGGESTING THAT SYMPATHETIC STIMULI ALSO CAN INFLUENCE WAT ENDOCRINE ACTIVITIES (FOR REVIEWS, SEE RefS. 24 AND 39). MORPHOLOGICALLY, THE SYMPATHETIC NORADRENERGIC NERVE FIBERS ARE LOCATED IN THE ADVENTITIA AND MEDIA OF WAT BLOOD VESSELS, MAINLY ARTERIES (13, 23, 50), FUNCTIONING TO ADJUST THE RATE OF WAT PERFUSION UNDER DIFFERENT PHYSIOLOGICAL CONDITIONS. AT A MORE PROXIMAL LEVEL TO THE FAT PAD, SYMPATHETIC NORADRENERGIC NERVE FIBERS USUALLY ARE ASSOCIATED WITH CAPILLARIES IN LOBULES AMONG WHITE ADIPOCYTES (13, 23, 40, 49, 50, 62). HISTORICALLY, THERE WAS SOME CONFUSION AS TO WHETHER WAT SNS INNERRATION ALSO OCCURRED TO OR NEAR THE WHITE ADIPOCYTES THEMSELVES. THIS WAS DUE TO THE TIGHT PACKING OF THE MATURE ADIPOCYTES IN ENERGY-REPLETIVE ANIMALS WITHIN THE FAT PAD SUPPORT MATRIX MAKING THE PARENCHYMA SPACE VIRTUALLY INVISIBLE EXCEPT AROUND THE VASCULATURE (50). THIS WAT PARENCHYMA NORADRENERGIC INNERRATION IS MORE READILY VISIBLE WHEN FASTING DECREASES WHITE ADIPOCYTE SIZE (50). MOREOVER, AN ADDITIONAL FACTOR THAT INCREASES THE SENSITIVITY OF THE WAT SYMPATHETIC NERVES IN THE PARENCHYMA ASSOCIATED WITH FASTING IS THE MARKED INCREASE IN THE DENSITY OF THE SYMPATHETIC INNERRATION TRIGGERED BY THIS LIPID DEPLETING STATE (22). THE PLASTIC ADJUSTMENT OF THE SUPPLY OF SYMPATHETIC NERVES TO WAT LIKELY FUNCTIONS TO FACILITATE THE RELEASE OF NE FROM PARENCHYMA SYMPATHETIC NERVE TERMINALS AS WELL AS INCREASING ITS DIFFUSION TO ADJACENT ADIPOCYTES TRIGGERS ADIPOCYTE LIPOLYSIS VIA β-ADRENOCEPTOR ACTIVATION. WAT DEPOTS ALSO ARE INNERRATED BY FIBERS POSSESSING NEuropeptides, MOST OF WHICH CONTAIN NEuropeptide Y (NPY) AND MAINLY ARE FOUND AROUND ARTERIES (13, 23). IN LABORATORY RAT RETROPERITONEAL AND EPIDIDYMAL WAT (RWAT AND EWAT, RESPECTIVELY), THE VAST MAJORITY OF NPY-IMMUNO-

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reactive (ir) nerve fibers also contain NE (22), suggesting that they belong to the SNS supply to WAT blood vessels. Finally, WAT has a provision of sensory nerves (16) that are capsaicin-sensitive and are immunoreactive for calcitonin gene-related peptide (CGRP) and substance P (23, 48, 49). These sensory nerves are part of afferent projections to brain sites across the neural axis, as revealed recently in pilot studies using the anterograde transneuronal tracer, the H129 strain of the herpes simplex virus-1 (Song CK and Bartness TJ, unpublished observations). The functional significance of these sensory nerves is not precisely known, although sensory innervation of rat periovarian adipose depot affects the recruitment of visceral brown adipocytes during cold acclimation (19), whereas in Siberian hamster EWAT, they appear to inform the central nervous system (CNS) of the presence or size of these WAT pads (48). Collectively, the existence of WAT sympathetic and sensory nerves is indisputable.

The transneuronal viral tract tracer, pseudorabies virus (PRV), has been used to identify the SNS outflow from brain to WAT in Siberian hamsters and laboratory rats (4, 9, 47) including indications of the receptors contained on these neurons (51, 53). Conclusions drawn from the results of a viral tracing study in laboratory rats indicated to some (26) that visceral and subcutaneous WAT also is provided with parasympathetic (PSNS) innervation. Specifically, it was reported that following local surgical denervation of WAT (to spare the purported parasympathetic nerves), subsequent PRV injections into these fat pads resulted in PRV-infected neurons in traditionally-recognized PSNS brain stem sites – the dorsal motor nucleus of the vagus (DMV) and the nucleus ambiguus (26). In the same report (26), other animals received selective local surgical parasympathetic WAT denervation (ostensibly sparing the sympathetic innervation) and subsequently WAT catabolic responses were enhanced (e.g., reductions in insulin-mediated glucose and free fatty acid uptake, increases in hormone-sensitive lipase activity). Although these data are intriguing, they are surprising in that previous studies showed no biochemical support for WAT parasympathetic innervation in terms of components of the primary PSNS postganglionic neurotransmitter, acetylcholine (2, 3). In addition, the results might be questioned because the ability to selectively surgically denervate the sympathetic or parasympathetic neural provisions to WAT seems extraordinary and because there was no attempt to localize markers of parasympathetic nerves in the WAT (26). It also is unclear why denervation of one component of the autonomic nervous system (SNS) would enhance visibility of the origins of another component (PSNS) using a viral tract tracer (see Discussion for details).

Therefore, the purpose of the present series of experiments was to test more rigorously and perhaps extend the notion of the parasympathetic innervation of WAT, as well as to characterize the anatomical distribution of this parasympathetic innervation and determine the biochemical phenotype of these nerves. This was accomplished by assaying a variety of WAT depots from laboratory rats and normal and obese mice using demonstrated markers of PSNS innervation. In a different neuroanatomical approach, we first chemically denervated the SNS innervation of WAT using local injections of the catecholaminergic neurotransmitter 6-hydroxydopamine (6-OHDA) into Siberian hamster inguinal WAT (IWAT) to destroy the sympathetic innervation and spare any PSNS innervation. This was followed 1 wk later with injections of the PRV to label specifically any parasympathetic nerves.

**MATERIALS AND METHODS**

**Experiment 1: Immunohistochemical Markers of Parasympathetic Nerves**

**Animals.** Sprague-Dawley male rats and C57BL normal and ob/ob male mice were purchased from Harlan Italy (San Pietro al Natisone, Italy) and had free access to food (65% carbohydrates, 11% fat, 24% protein, wt/wt) and water. Lights were on for 12 h daily (0700–1900) and all animals were kept at a temperature of 22°C. All procedures and care were in accordance with Italian institutional guidelines.

At the time of death, three rats for each condition (growing, adult, and obese) were anesthetized [100 mg/kg ketamine (Ketavet; Farmaceutici, Gellini, Aprilia, Italy) in combination with 19 mg/kg xylazine (Rompum; Bayer, Leverkusen, Germany)] and perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. RWAT and EWAT, as well as IWAT and control tissues (see Immunohistochemistry) were dissected and postfixed by overnight immersion in the same fixative. Samples were then dehydrated and paraffin-embedded.

**Immunohistochemistry.** Immunoreactivity was assessed in 3-μm-thick sections by using the avidin-biotin-peroxidase (ABC) method. For each of the PSNS markers (this section), the immunohistochemical analysis was performed for each WAT depot on at least five sections spaced 300–400 μm. Sections were deparaffinized in xylenes, hydrated, and incubated with 0.3% H2O2 in methanol for 30 min at room temperature to block endogenous peroxidase, washed in PBS (2 × 15 min) and incubated in 1:75 vol/vol normal goat serum (Vector Laboratories, Burlingame, CA) in PBS for 20 min at room temperature to block nonspecific sites. Sections were incubated overnight at 4°C with the primary antibody against vesicular acetylcholine transporter (VAChT; polyclonal rabbit, Sigma, St. Louis, MO) at a dilution of 1:500, with the primary antibody against vasoactive intestinal peptide [VIP; polyclonal guinea pig, (Peninsula Laboratories, San Carlos, CA)] at a dilution of 1:3,000 or with the primary antibody against neuronal nitric oxide synthase [nNOS; polyclonal rabbit (Cayman Chemical, Ann Arbor, MI)] at a dilution of 1:800, washed with PBS (2 × 15 min); incubated in 1:200 vol/vol biotinylated IgG anti-rabbit goat serum (Vector; VACHT and nNOS schedules) or 1:200 vol/vol biotinylated IgG anti-guinea pig goat serum (Vector; VIP schedule) in PBS for 30 min at room temperature; washed with PBS (2 × 15 min); incubated in ABC Elite reagent (Vector) in PBS for 1 h at room temperature; washed with PBS (2 × 15 min); incubated in 0.02% H2O2 and 0.075% diaminobenzidine (Sigma) in 0.05 M Tris buffer (pH 7.6), kept for 5 min in a dark room and finally rinsed in distilled water. Sections were counterstained with hematoxylin, dehydrated and mounted in Entellan. The ability of the antibodies to detect VAChT- and VIP- and nNOS-containing nerves in formalin-fixed paraffin-embedded tissues was evaluated in sections (obtained according to the procedure described above) of tissues known to contain the antigens, such as submandibular gland and heart for VAChT-positive nerves, small intestine for VIP-positive nerves, and stomach for nNOS-positive nerves. Negative controls were obtained in each instance by omitting the primary antibody and using preimmune instead of primary antisera.

The images were stored as TIFF files. Brightness and contrast of the final images were adjusted using the Photoshop 6 software (Adobe Systems, Mountain View, CA)

**Immunofluorescence and confocal microscopy.** Immunofluorescence was performed on paraffin embedded sections obtained according to the procedure described above. After deparaffinification and hydration, sections were incubated in 1:75 vol/vol normal donkey serum (Jackson ImmunoResearch, West Grove, PA) in PBS for 20 min at room temperature to block nonspecific sites. Sections were
incubated overnight at 4°C with the mixture containing the primary antibodies against protein gene expression product 9.5 (PGP9.5) (rabbit polyclonal; Ultraclone, Isle of Wight, UK) and tyrosine hydroxylase (TH; sheep polyclonal; Chemicon, Temecula, CA) at dilutions of 1:500 and 1:300, respectively; washed with PBS (2 × 15 min); and incubated in 1:100 vol/vol FITC donkey anti-rabbit (Jackson) and tetramethylrhodamine isothiocyanate (TRITC) donkey anti-sheep (Jackson) antibodies in PBS for 30 min at room temperature. Sections were subsequently washed with PBS (2 × 15 min), air-dried, and coverslipped using Vectashield mounting medium (Vector). Fluorescence was detected with a Leica TCS SP2 spectral confocal microscopy (Leica Microsystems, Vienna, Austria) equipped with krypton/argon and two different helium lasers. Fluorescein isothiocyanate (FITC) and TRITC were excited with the 488 and 543-nm lines, respectively, and imaged separately. We examined at least five sections for each depot spaced 300–400 microns. Sections were viewed in a Leica DM-6000 microscope with a × 63 plan-apochromat objective and 1.4 numerical aperture. Images (512 × 512 pixels) were obtained sequentially from two channels using a pinhole of 102.00 µm and stored as tagged image file format (TIFF) files. Brightness and contrast of the final images were adjusted using the Photoshop 6 software (Adobe Systems).

**Experiment 2: Local Chemical Sympathetic Denervation and PRV Injection into WAT Depots**

**Animals.** Adult male Siberian hamsters ~3.5 mo old were obtained from our breeding colony, the lineage of which has been described recently (9). Hamsters were exposed to a long day photoperiod (16:8-h light-dark cycle; lights on at 0200 h) from birth and kept at 21 ± 2°C. PMI Rodent Diet no. 5001 (Purina, St. Louis, MO) and tap water were provided ad libitum throughout the experiment. Hamsters were single housed 1 wk before IWAT sympathetic denervations. All procedures were approved by the Georgia State University Institutional Animal Care and Use Committee and are in accordance with Public Health Service and United States Department of Agriculture guidelines.

**Sympathetic denervations.** Two sets of animals were used. In the first, one IWAT depot of hamsters was sympathetically denervated via local injections of the catecholaminergic toxin, 6-OHDA (n = 9) or the vehicle (n = 4), and 7 days later injected with PRV (as described below) for examination of spinal cord and brain infections. In a subsequent experiment, we tested whether trauma to the autonomic nervous system triggers a slowing of viral infection/transition that might lead to a failure to see spinal cord or brain PRV infections. This was done in light of suggestions that systemic 6-OHDA injections could nonspecifically thwart the progress of PRV through neural circuits (Oldfield B, personal communication). Therefore, additional groups of animals were tested where 6-OHDA, or the vehicle, was injected into the same IWAT pad (as in the first group; n = 4) or in the ipsilateral interscapular brown adipose tissue (IBAT; n = 2). Components of the sympathetic chain (i.e., T13 and stellate ganglia) were included in the histological analyses to determine whether the PRV was stalled in its transit either because of the localized chemical denervation, or by a more generalized insult to the SNS.

**Animals were anesthetized with pentobarbital sodium (50 mg/kg) and the target incision area over the rear haunch area was shaved and wiped with 70% ethanol. An incision was made at the dorsal hind limb of the animal and lateral to the spinal column that continued rostrally and then ventrally to the ventral hind limb. Care was taken with the depth of the incision so as to not damage the underlying fat pad and vasculature. The application of 6-OHDA (Sigma) was performed as described previously for the purpose of sympathetic denervation of rat testes (31). Briefly, once the IWAT pad was exposed, a series of injections of 6-OHDA was made using a 10-µl microsyringe at 40 loci within the fat pad (4 mg in 100 µl vehicle; n = 10) to evenly distribute the sympathetic neurotoxin. In the control animals, IWAT was unilaterally injected similarly, but with vehicle only (n = 4). The contralateral fat pads in both treatment groups were left intact and served as a within-animal control. The incision was closed with sterile wound clips and nitrofurazone powder was applied to minimize sepsis. The animals were then transferred to clean cages and recovered for 1 wk before PRV injections.

**PRV injections.** One week after 6-OHDA or vehicle injections, PRV was injected into the same IWAT, or the ipsilateral IBAT as described previously (4, 5). In brief, once the IWAT pad was exposed, a series of injections of PRV 152 (a generous gift of Lynn Enquist, Princeton, NJ) was made using a 1.0-µl microsyringe at five loci within the fat pad (3 × 10⁶ pfu/ml; 125 nl/loci) to evenly distribute the virus. The incision was closed with sterile wound clips and nitrofurazone powder was applied to minimize sepsis. The animals were then transferred to clean cages and survived for 6 days, the postinoculation survival time for infection to reach the rostral forebrain from these fat pads in this species (4).

Six days after PRV injections (13 days after sympathetic denervations), both IWAT pads were removed under pentobarbital sodium (50 mg/kg ip) anesthesia, snap-frozen in liquid nitrogen and stored in −80°C until processed for NE content by HPLC with electrochemical detection (see below). The animals were then given an overdose of pentobarbital sodium (300 mg/kg ip) and perfused transcardially with 0.1 M sodium azide. The brains were sliced at 30 µm using a freezing stage sliding microtome along the coronal plane and the ganglia were sliced on a cryostat (Leica CM3500S) at 20 µm thickness, thaw-mounted onto Superfrost Plus slides (Fisher Scientific) and processed for immunohistochemical detection of PRV, TH, and choline acetyltransferase (ChAT).

**WAT NE content.** SNS denervation of the excised fat pads was verified by measuring NE content using reverse-phase HPLC with electrochemical detection, as we have done previously (9). In brief, a sample of IWAT (~100 mg) was thawed and homogenized in a solution containing 10 ng dihydroxybenzylamine (internal standard) in 0.2 M perchloric acid with 1 mg/ml ascorbic acid (PCA-AA). After centrifugation at 7,500 g for 10 min, catecholamines were extracted from the homogenate with alumina and eluted into PCA-AA. Catecholamines were assayed using a HPLC system (ESA; Bedford, MA) with electrochemical detection (Coulombic II). The mobile phase was Cat-A-Phase II, the column was a C18 reverse-phase column, and the detector settings were as previously described (62).

**Immunohistochemistry for single-label PRV.** Brain sections were rinsed multiple times in PBS (0.1 M; pH 7.4), dehydrated in ascending concentrations of ethanol, delipidated with chloroform, rehydrated in descending concentrations of ethanol, rinsed in PBS and then incubated in the primary antibody (Rb132; 1:10,000) (a generous gift of Lynn Enquist, Princeton, NJ) overnight. The secondary antibody (goat anti-rabbit; 1:500; Vector) was applied for 2 h, and then the sections were placed in ABC (Vector) for 1 h. The specific labels were detected using diaminobenzidine (0.1 mg/ml) as the chromogen in the presence of peroxide (0.0025%). All steps in the immunohistochemistry procedure were performed at 22°C. The sections were mounted onto gelatin-coated slides and air-dried. The sections were then counterstained with cresyl violet and coverslipped. Staining for PRV was observed from the level of the preptic area rostrally and through the brain stem caudally using an Olympus BX41 microscope. Images were captured digitally with an Olympus DP70 camera and acquired using Adobe Photoshop (version 6.0). A mouse brain atlas was used as a guide to identify brain regions (36).

**PRV-infected cells in ganglia were detected similarly to that in brain except that the incubation times were increased for the primary antibody (2 day), secondary antibody (1 day), and ABC (1 day). All
other steps in the immunohistochemistry procedure including concentrations and incubation diluent and temperatures were unmodified.

**Immunohistochemistry and quantification for double-label PRV and TH or ChAT in DMV**

**PRV and TH.** Brain stem sections at the level of the DMV were incubated sequentially in mouse anti-TH (1:5,000; ImmunoStar Hudson, WI) for 3 days, goat anti-mouse (Vector; 1:500) for 1 day and avidin-conjugated Texas Red (1:500; Vector) for 2 h. Specific labels for TH were visualized with a red fluorophore filter and for the enhanced green fluorescent protein (EGFP) reporter of PRV 152 with a green fluorophore filter. Images were captured using DP70 digital camera and superimposed to visualize both green and red fluorescence from the same sections.

**PRV and ChAT.** Sections were first processed for the detection of ChAT by incubating in goat anti-ChAT (1:5,000, 4 days; Chemicon International, Temecula, CA), horse anti-goat (1:500, 1 day; Vector), and ABC (2 h). Once the specific label for ChAT above except that 1-Napthol (Sigma; 1 mg/ml) was used in the presence of H2O2 (0.006%) for a blue color to contrast against the brown diaminobenzidine (DAB).

**Quantification of single- and double-labeled PRV cells in the DMV cells.** Every sixth section was examined through the rostral-caudal extent of the DMV. Within the bilateral DMV of each section, the total number of PRV-infected cells was compared with the number of double-labeled cells, i.e., [PRV + TH]/PRV_total or [PRV + ChAT]/PRV_total. The numbers were then tallied across the sections and presented as percent ratios.

**Statistical Analyses**

Catecholamine content data were analyzed using one-way analysis of variance (NCSS, Kaysville, Utah). Post hoc analysis was performed using Duncan’s New Multiple Range Test (25). Differences were considered statistically significant if P < 0.05. Exact probabilities and test values were omitted for simplicity and clarity of the presentation of the results.

**RESULTS**

**Rat and Mouse WAT Does Not Show Evidence of Cholinergic Nerves**

Acetylcholine is the predominant transmitter of the PSNS postganglionic nerves (for review, see Ref. 28). The vesicular acetylcholine transporter (VACHT) resides in synaptic vesicles of cholinergic nerve terminals and is a reliable marker for detecting central and peripheral cholinergic nerve terminals (44). Using paraffin-embedded sections, we assayed the major WAT depots of adult rodents for VACHT immunoreactivity (ir), and also included rat submandibular gland (Fig. 1A) and mouse heart (Fig. 1B) as positive controls. Cholinergic nerves (VACHT-ir nerves) were never observed in immunostained sections obtained from several different levels of rat or mouse EWAT (Fig. 1C), RWAT, or IWAT. PSNS activation in WAT appears to increase insulin sensitivity and fat synthesis (26). Thus we speculated that PSNS cholinergic nerves could be better visualized in WAT under conditions of fat growth. Therefore, we examined WAT samples obtained from growing rats and mice during the 3rd and 4th wk of postnatal life and from leptin-deficient ob/ob obese adult mice. In these animals too, RWAT, EWAT, and IWAT were devoid of VACHT-ir nerves (data not shown).

**Fig. 1.** Immunohistochemistry against vesicular acetylcholine transporter shows the presence of cholinergic fibers in rat submandibular gland (A, arrows) and mouse heart (brown, B), taken as positive controls, but fails to detect any cholinergic, putatively parasympathetic nerve in the rat epididymal (C) fat. V, blood vessel. Bars: A and B = 55 μm; C = 30 μm.
Rat and Mouse WAT Does Not Show Evidence of VIP- or nNOS-Containing Nerves

Parasympathetic postganglionic cholinergic nerves innervating peripheral tissues often contain VIP (28); moreover, VIP also may be the only known neurotransmitter in a subset of parasympathetic noncholinergic nerves (28). Nonadrenergic, noncholinergic nerves belonging to the vagal PSNS contain nNOS in the gastrointestinal tract (8, 13). Thus, to confirm the absence of cholinergic nerves in WAT and, at the same time, establish the presence of a possible VIP- or nNOS-ir parasympathetic supply to it, we performed VIP and nNOS immunohistochemistry on our WAT samples and on small intestine (Fig. 2, A and stomach (Fig. 2, B and C) tissues taken as positive controls. VIP- or nNOS-ir nerves were never found in the RWAT (Fig. 2D), EWAT, or IWAT of adult or growing rats or mice, nor in these WAT pads of ob/ob obese mice.

Double-Labeling Experiments Show that Nonnoradrenergic Nerve Fibers Are Uncommon In Rat Or Mouse WAT

To provide conclusive evidence for or against the presence of a substantial nerve supply to WAT other than the sympathetic one, WAT nerves were double-stained by immunofluorescence using antibodies against PGP9.5, a pan-nerve marker (56, 61), and TH, a specific marker for sympathetic noradrenergic nerves (17). Using confocal microscopy of sections from RWAT, EWAT, and IWAT of adult or growing rats and mice and of ob/ob obese mice there were very few fibers innervating blood vessels and the WAT parenchyma that were not noradrenergic (i.e., that also did not show TH-ir; Fig. 3). A quantitative estimate of parenchymal noradrenergic nerves was performed on sections (5 for each depot) of RWAT and EWAT of normal animals. In these conditions, only about 2–3% of the total number of nerve fibers running among the adipocytes was noradrenergic. By contrast, nonnoradrenergic nerve bundles were found both in the interlobular space together with large arteries and veins and into fat lobules, especially in the retroperitoneal depot (Fig. 2).

Sympathetic Denervation Decreased NE Content and Blocked CNS PRV Infections

NE contents of IWAT pads were measured to verify sympathetic denervations. Results are shown as percent decrease in NE of the 6-OHDA or vehicle treatment with respect to the contralateral, intact control IWAT {i.e., %decrease = 100[(NE ng per g intact IWAT) − (NE ng per g treated IWAT)]/(NE ng per g intact IWAT)}. NE content was significantly (P < 0.05; Fig. 4) decreased in IWAT after localized 6-OHDA injections (n = 9) compared with vehicle treatment (n = 4).

None of the animals exhibited overt signs of illness for the 6-day postinoculation period. The CNS infection pattern in the intact control group was virtually identical with that found previously when PRV 152 or its parental Bartha’s K strain was injected into the same tissue (4, 47, 51, 53). Forebrain PRV infections (Fig. 5A) were in various areas including the medial preoptic area, hypothalamic paraventricular nucleus, arcuate nucleus, dorsomedial nucleus, lateral hypothalamus, and the suprachiasmatic nucleus. PRV infections in the midbrain (data not shown) were concentrated in the periaqueductal gray and in the pedunculopontine tegmental nuclei. Brain stem PRV infection (Fig. 5, B and C) was found bilaterally in the nucleus of the solitary tract, paramedian reticular nucleus, intermediate reticular nucleus, rostroventrolateral reticular nucleus, lateral paragigantocellular nucleus, and ventral spinocerebellar tract, as well as in the raphe pallidus, raphe obscurus, raphe magnus, and area postrema. There also were PRV-infected cells scattered in area 10 (i.e., DMV; Fig. 5C), particularly along the outer edges, but occasionally within the nuclei. In stark contrast, we found no PRV infections in the forebrain (Fig. 6A),
midbrain, brain stem (Fig. 6, B and C), spinal cord, or T13 sympathetic ganglia (Fig. 7) when PRV was injected into IWAT previously injected with 6-OHDA to induce a chemical sympathetic denervation.

To test for potential slowing or cessation of infection due to sympathetic denervation-induced trauma (Oldfield B, personal communication), we injected IWAT with 6-OHDA or the saline control vehicle before virus inoculation. In animals where the vehicle was first injected into WAT followed subsequently by PRV injections, infection was found in the T13 sympathetic ganglion (Fig. 8), but not the stellate ganglion (Fig. 7C), ipsilateral to the PRV-injected IWAT. In hamsters with IWAT first injected with 6-OHDA and subsequently with PRV, no T13 sympathetic ganglion infections occurred (Fig. 7). As a further test for potential slowing or cessation of infection due to denervation-induced nonspecific effects on PRV transport, we injected 6-OHDA into IWAT of some hamsters, as before, but then injected PRV into the ipsilateral IBAT pad. Animals with 6-OHDA-injected IWAT and subsequently injected with PRV in the ipsilateral IBAT had PRV infection in the ipsilateral stellate ganglion (Fig. 9A), but not in the contralateral stellate (Fig. 9B) or in the T13 sympathetic ganglia (Fig. 9C). In these latter animals, the infection pattern in the brain resembled that from our previous animals that were injected in IBAT with PRV, but not treated with 6-OHDA (5).

In a small pilot experiment, we injected PRV into IWAT and tested whether the DMV (area 10) infections were in TH (Fig. 10) or ChAT (Fig. 11) synthesizing neuronal somata. PRV-infected DMV cells were unilateral to the injected IWAT pad. Of the PRV-infected cells in the DMV, ~30% (56/183 PRV-ir cells) and ~9% (12/132 PRV-ir cells) were colocalized with TH (Fig. 8A) and ChAT (Fig. 8B), respectively.

**DISCUSSION**

The results of the present study question the extent of the PSNS innervation of WAT (26) based on classic morphological identification of proven markers for parasympathetic nerves in peripheral tissues. That is, we found an absence of VAChT-ir, a general marker of PSNS innervation (1, 43, 45), as well as a lack of VIP- and nNOS-ir, thought to be the only known neurotransmitters in a subset of parasympathetic noncholinergic nerves (28), in several WAT depots (RWAT, EWAT, and IWAT) of laboratory rats, a standard laboratory mouse strain (C57BL) and a genetically-based obese mouse strain (ob/ob).

In addition, in an attempt to label exclusively the possible PSNS innervation of WAT (26) based on classic morphological identification of proven markers for parasympathetic nerves in peripheral tissues. That is, we found an absence of VAChT-ir, a general marker of PSNS innervation (1, 43, 45), as well as a lack of VIP- and nNOS-ir, thought to be the only known neurotransmitters in a subset of parasympathetic noncholinergic nerves (28), in several WAT depots (RWAT, EWAT, and IWAT) of laboratory rats, a standard laboratory mouse strain (C57BL) and a genetically-based obese mouse strain (ob/ob).
WAT vasculature and parenchyma that were labeled with PGP9.5 (pan-nerve marker) also contained TH, attesting to the pervasiveness of WAT SNS innervation and lack of, or meagre, PSNS innervation. Taken together, the present data support the earlier biochemical studies showing no acetylcholine esterase, the widely distributed enzyme involved in postsynaptic catabolism of acetylcholine, in WAT (2, 3), indicating that WAT is not, or is sparsely at best, innervated by the PSNS.

Dual innervation of tissues by the SNS and PSNS is the rule, with a few exceptions [e.g., lacrimal gland, the bronchi, most blood vessels (30)]. The lack of PSNS innervation of WAT,

Fig. 5. Photomicrograph illustrating diaminobenzine (DAB; brown) labeled pseudo-rabies virus (PRV) infected neurons in the forebrain (A) and the brain stem (B and C) when PRV was injected into vehicle-treated IWAT. The nucleus of the solitary tract (sol) and area 10 [10; vagal dorsomotor nucleus (DMV)] in B is magnified in C for enhanced visibility of the infection pattern. PVN, paraventricular hypothalamus; SCh, suprachiasmatic nucleus; ZI, zona incerta; OC, optic chiasm; dorsal motor nucleus of the vagus nerve; 12, hypoglossal nucleus; ROb, raphe obscurus; Irt, intermediate reticular nucleus; P Mn, paramedian reticular nucleus; RVL, rostroventrolateral reticular nucleus; VSC, ventral spinocerebellar tract.

Fig. 6. Photomicrograph illustrating forebrain (A) and brain stem (B and C) of an animal when PRV was injected into 6-OHDA-denervated IWAT. The sol and area 10 (DMV) shown in Fig. 6B is magnified in Fig. 6C. RPa, raphe pallidus; Xi, xiphoid nucleus; 3V, 3rd ventricle.
therefore, does not afford it the fine functional control that tissues such as the iris of the eye or the heart have with both branches of the autonomic nervous system contributing to their innervation (30). The initial report of PSNS innervation (26), therefore, appeared to provide WAT with an opposing neural counterpart to the initiation of lipid mobilization by the SNS innervation that can be triggered by cold exposure (19), fasting (33), or estradiol treatment (27) in laboratory rats, or short-day exposure in Siberian hamsters (62). Thus it would seem that lipid accumulation, the opposing response to lipid mobilization, is accomplished by a combination of decreases in sympathetic drive/adrenergic signaling and increases in humoral substances that promote lipid storage, such as insulin and glucocorticoids (34), and likely little or no influence by the PSNS.

In a recent attempt to visualize the possible PSNS innervation of WAT, only the sympathetic innervation was surgically denervated sparing the purported parasympathetic innervation (26), a remarkable feat given the identical outward appearance of sympathetic and parasympathetic nerves. In addition, we are unaware of anatomically separated sympathetic and parasympathetic neural provisions to WAT pads. Nevertheless, by doing so, the spared PSNS could then, in principle, be labeled with subsequent injections of PRV. This approach yielded extraordinarily dense, bilateral PRV-infected neurons in the DMV (26), among other areas. In the same study, functional evidence supporting the PSNS innervation of WAT was suggested, using an opposite approach. That is, WAT was ostensibly, selectively, and specifically surgically vagotomized (parasympathectomized) and this yielded reductions of insulin-mediated glucose and free fatty acid uptake (26). In addition, increases in the activity of a principal intracellular mediator of lipolysis, hormone-sensitive lipase, occurred in these surgically parasympathectomized WAT pads (26). Together, these functional data suggested to the authors that with the surgical removal of the PSNS innervation of WAT, the SNS innervation was unopposed, and, hence, catabolic responses were triggered (e.g. lipolysis). Thus the PRV-infected DMV and nucleus ambiguus cells combined with these functional data suggested to the authors not only the presence of parasympathetic innervation of WAT, but an anabolic role of this innervation (26) (but see also Refs. 6, 8, and 53). An alternative hypothesis for the functional data that cannot be ruled out from the Kreier et al. (26) data is that the denervation altered blood flow to the tissue producing the functional responses attributed to the parasympathetic drive in the absence of sympathetic innervation.

In terms of neuroanatomical support for the claim of PSNS innervation of WAT by Kreier et al. (26), we found no evidence of a PSNS supply to WAT in the present study. Indeed, using immunohistochemical detection of specific nerve markers for the known parasympathetic neurotransmitters (VACht, VIP, nNOS) and assaying all major WAT depots of normal adult mice, obese mice and laboratory rats, there was no VACht-, VIP-, or nNOS-ir nerves in any fat pad from all of these animals. Most importantly, visualization of the entire nerve supply to WAT blood vessels and lobules with a pan-nerve marker (PGP9.5; Refs. 56 and 61) showed very few fibers that were not noradrenergic (i.e., that did not show TH immunoreactivity and therefore that were not sympathetic; present findings and Ref. 22). It is possible, however, that the PSNS innervation of WAT is extremely meagre and localiza-
tion of parasympathetic markers in tissues that are relatively lightly innervated compare with other peripheral tissues (e.g., heart, adrenal) would be akin to finding the proverbial “needle in a haystack.” Given the large number of WAT pads and slices

Fig. 8. Photomicrograph illustrating PRV infection within the T13 ganglion ipsilateral to the IWAT injected with saline and followed by PRV 1 wk later (A). Similar infection was not found in the contralateral T13 (B) nor the ipsilateral stellate ganglion (C).

Fig. 9. PRV infection was found in the stellate ganglion (A) when injected into interscapular brown adipose tissue in animals where the ipsilateral IWAT was injected with 6-OHDA to produce a sympathetic denervation. Similar infection was not found in the contralateral stellate ganglion (B) nor in the T13 ipsilateral to the denervated IWAT (C).
per pad examined without a single incidence of positive staining for a PSNS marker, this seems unlikely but not improbable. Collectively, these findings exclude the presence of an important nerve supply to WAT other than sympathetic innervation.

According to classical autonomic physiology, PSNS post-ganglionic nerve fibers originate from parasympathetic ganglia located near to, or contained within, the target organ. Notably, ganglia were never observed near to, or within, the adipose depots in rodents (Giordano A and Cinti S, unpublished observations) which begs the question as to the location of these ganglia. For that we have no answer, nor is their existence or location addressed by the proponents of the PSNS innervation of WAT (26).

For technical reasons (14), the present study was performed on paraffin-embedded samples in which a certain amount of immunoreactivity may have been lost because of stronger tissue fixation and of high-temperature wax infiltration, although this did not affect VAChT-, VIP-, or nNOS-ir in the positive control tissues (submandibular gland, heart, intestine and stomach) processed identically to the WAT samples. We estimate the loss of immunoreactivity on the basis of comparisons between paraffin-embedded and standard fixation to be ~15–20% and therefore do not feel this loss could explain the lack of VAChT-, VIP-, or NOS-ir in WAT. The vast majority of vascular and parenchymal WAT nerves, however, were noradrenergic, as we saw previously in the more immunoreac-

Fig. 10. PRV-infected cells in the brain were colocalized with TH (A). PRV infection in the brain stem was visualized by the green fluorescent protein reporter (B), and TH was immunohistochemically labeled with Texas Red (C). D: images in B and C are superimposed and enlarged such that PRV-infected cells, also labeled for TH (PRV + TH = yellow), are better visible. Of the 23 PRV-infected cells found in area 10 (DMV), ~30% also were labeled for TH.
Given that Kreier et al. (26) injected PRV unilaterally, as we ever, the Kreier study (26) used male rats. More importantly, ambiguus cells, and this is decreased by vagotomy (20) [how-

tination of the ovaries, in which PRV injections label nucleus

tissues is missing, with the possible exception of the innerva-

tion of unequivocal nucleus ambiguus projections to abdominal

abdominal WAT. Secondly, to our knowledge, the demonstra-

any previously described vagal nerve bundles projecting to

supposed by Kreier et al. (26) who reported significant DMV

neurons represent PSNS outflow from brain to WAT as was

55), so it would be tempting to assume that these labeled

virus-infected cells within the DMV. The DMV traditionally is

known as a locus for vagal motor neurons (e.g., Refs. 29, 54,

vated by these DMV efferents.

For the nucleus ambiguus infections and intense bilateral label-

ing of DMV cells. We did find some DMV infection in the

present study, with a small number and percentage of ChAT +

PRV-ir and a larger number and percentage of TH + PRV-ir
cells in the DMV, the latter likely catecholaminergic vagal
visceromotor neurons (42). The latter data alone suggest the

possibility of vagal innervation of WAT, but are at odds with

the complete absence of labeling for established markers of

parasympathetic nerves in WAT we found in the present study.

One possible resolution to these discrepant data within the

present study is that because there are so few cells labeled in

the DMV after WAT PRV injections, at least in our hands, this

may lead to a correspondingly few number of postganglionic
ganglionic vagal nerves invading WAT making it difficult, if not nearly

impossible to locate them. If the DMV-infected cells innervate

WAT via the vagus, their apparent small number questions their

importance functionally. Obviously, further studies are

warranted, such as those identifying the preganglionics innerv-

ated by these DMV efferents.

Finally, because we are unable to discern sympathetic from

parasympathetic nerves using a dissecting microscope, we

approached the strategy of Kreier et al. (26) to destroy the SNS

innervation of WAT and thus spare the putative PSNS innerva-

tion, but did so by local injections of the catecholaminergic

nerve toxin 6-OHDA, followed 1 wk later by injections of PRV
to label any remaining nerves, whether parasympathetic or

sympathetic. We know the 6-OHDA was effective in destroy-
ing the sympathetic innervation in IWAT because NE content

was significantly reduced compared with vehicle-injected

IWAT. In our experience (41), and in the experience of others

(15, 46, 57), although the immediate effect of 6-OHDA is to

produce substantial NE depletions, there is a relatively quick

return of NE content likely due to sprouting of nondamaged

neurons into areas previously innervated. Because we found no

infections in the sympathetic ganglia, spinal cord, or brain,

arguments that the sympathetic denervation appeared far from

the present study, we found PRV-labeled cells in the DMV

only unilateral to the side of the IWAT injection, although the

number of infected cells here appears considerably less than

depicted in the microphotograph by Kreier et al. (26) and we

found no nucleus ambiguous infected cells [or in our previous

work (4, 9, 47, 53)]. There are many reasons for the differences in

PRV-ir DMV cells between these two studies and we offer

several here. The considerable difference between the number of

PRV-infected neurons between these studies simply could be

because of species differences (laboratory rats vs. Siberian

hamsters here), although we found no striking differences in

PRV-infected cells across the neuroaxis between these two

species in our original report of PRV-labeled SNS outflow
circuits to WAT (4) including the DMV. Alternatively, as the

authors suggest (26), the intense PRV-labeling of DMV cells in

their study may be because of forcing the virus through the

residual parasympathetic nerves after their selective local sur-

gical sympathetic, but not parasympathetic denervation of

WAT (26). The viral load would be different with the absence

of the sympathetic innervation, although viral concentration

and degree of innervation seem more important than load per

se (Ref. 12 and for review, see Refs. 11, 52). Given that no

controls were performed for viral leakage from the injection

site in the Kreier et al. study (26), it seems possible that the

PRV spread to other tissues intraperitoneally, thus accounting

for the nucleus ambiguous infections and intense bilateral label-

ing of DMV cells. We did find some DMV infection in the

present study, with a small number and percentage of ChAT +

PRV-ir and a larger number and percentage of TH + PRV-ir
cells in the DMV, the latter likely catecholaminergic vagal
visceromotor neurons (42). The latter data alone suggest the

possibility of vagal innervation of WAT, but are at odds with

the complete absence of labeling for established markers of

parasympathetic nerves in WAT we found in the present study.

When the CNS outflow from brain to WAT was labeled for

the first time using the PRV (4), we found some scattered

virus-infected cells within the DMV. The DMV traditionally is

known as a locus for vagal motor neurons (e.g., Refs. 29, 54,

55), so it would be tempting to assume that these labeled

neurons represent PSNS outflow from brain to WAT as was

supposed by Kreier et al. (26) who reported significant DMV

and nucleus ambiguous infected cells. First, we are unaware of

any previously described vagal nerve bundles projecting to

abdominal WAT. Secondly, to our knowledge, the demonstra-

tion of unequivocal nucleus ambiguous projections to abdominal

tissues is missing, with the possible exception of the innerva-

tion of the ovaries, in which PRV injections label nucleus

ambiguous cells, and this is decreased by vagotomy (20) [how-

over, the Kreier study (26) used male rats]. More importantly,
given that Kreier et al. (26) injected PRV unilaterally, as we
did originally (4) and here also, their reported bilateral labeling
of the DMV is at odds with its traditional unilateral vagal
innervation of peripheral tissues in rodents (18, 32, 35, 38). In

tive cryosections of slimmed adipose tissue obtained from

fasted rats (22). Thus the absence of immunohistochemical
evidence of parasympathetic nerves in WAT of rats and mice

along with the lack of biochemically detectable acetylcholine

esterase in laboratory rat WAT (2, 3) appear to make it unlikely

that WAT contains appreciable PSNS innervation.

Fig. 11. PRV infection (1-Napthol, blue) in the brain was colocalized with choline acetyltransferase (DAB, brown) after injection into IWAT. The left brain stem area 10 (DMV) shown in A is enlarged in B for enhanced visibility.
complete (only an ~60% decrease) at death are irrelevant. We also are sensitive to the possibility that trauma induced by 6-OHDA could have slowed the progression of the virus through the nervous system (Oldfield B, personal communication), but the absence of sympathetic ganglia infections counters this possibility. Clearly, local treatment with 6-OHDA blocked transport of PRV into the sympathetic chain, spinal cord, and brain, whereas vehicle injections did not. These data, when combined with the lack of immunohistochemical data showing markers of WAT PSNS innervation, provide converging neuroanatomical evidence that supports, at best, meagre PSNS innervation of WAT, despite the presence of sparse DMV multisynaptic connections to WAT.

White adipocytes share many developmental, morphological, and biochemical features with brown adipocytes, the function of the latter being to produce heat. It should be noted, however, that brown and white fat depot are never “pure,” as both cell types are found in each depot, at least in rodents (11). The prevalent metabolic function of these depots (i.e., thermogenesis or lipid storage) depends on the relative number of white and brown adipocytes, which, in turn, is affected by strain, diet, and environmental temperature. In a previous investigation on laboratory rat BAT, we detected a clear cholinergic nerve supply, as evidenced by VAChT-ir at the vascular and parenchymal levels, but only mediastinal BAT, and not in IBAT or perirenal BAT (21); moreover, this innervation was consistently modulated by both cold and fasting (21). Thus it appears that of all rodent fat depots, mediastinal BAT provides the best example of putative PSNS innervation of adipose tissue, but verification with tract tracing studies and demonstration of its functional significance remains to be done for this BAT depot.

An interesting result of our nerve double-labeling experiments is that WAT depots contain large nonnoradrenergic nerve bundles accompanying large blood vessels and coursing through fat lobules. For technical reasons, we were unable to characterize these fibers phenotypically. WAT receives its own vascular-nerve peduncle at the hilus, such as occurs for the liver or kidney and is crossed by nerves directed to extraadipose structures. Thus another way to resolve the discrepancy between the present results and those of Kreier et al. (26) could be that their PRV injections were not restricted to the WAT pad itself and caused infection of these large nerves that could contain PSNS fibers that course through the WAT depots, but are not specifically directed at WAT blood vessels or at white adipocytes, although they would have to have nerve terminals in this area to pick up the PRV.

In conclusion, our efforts to detect and phenotype the parasympathetic nerves in WAT by immunohistochemistry failed, and by locally chemically denervating WAT, we blocked transport of the virus through all neural circuits as evidenced by an absence of infected cells in the sympathetic chain, spinal cord, or brain. These results agree with those of previous work (2–4, 53) and strongly argue that the only autonomic innervation of WAT is sympathetic. We did find, however, some labeling of DMV cells after PRV WAT injection in the present study and in our previous work in Siberian hamsters and laboratory rats (4, 9, 47, 53), but did not find PRV-infected cells in another traditionally classified PSNS site, the nucleus ambiguus, as reported by Kreier et al. (26). The lack of classic morphological evidence for PSNS innervation of WAT at the level of the pad is somewhat at odds with the PRV-infected DMV cells, although these were few in number and perhaps, therefore, also ultimately result in relatively few parasympathetic nerves terminating into WAT, making detection difficult at best. Such scant DMV projections to WAT also seem to question its physiological significance. Finally, the distinction between the central origins of the SNS and PSNS innervation of peripheral tissues has become blurred (for a review, see Ref. 37), including observations that single neurons are part of both the sympathetic and parasympathetic innervation of disparate peripheral tissues (60). Therefore, force-fitting neuroanatomical results into this autonomic dichotomy likely may hinder rather than advance our understanding of the trafficking of CNS outflow to peripheral tissues.

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