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Brain-Adipose Tissue Neural Crosstalk[1]

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Abstract

The preponderance of basic research on obesity focuses on its development as affected by diet and other environmental factors, genetics and their interactions. By contrast, we have been studying the reversal of a naturally-occurring seasonal obesity in Siberian hamsters. In the course of this work, we determined that the sympathetic innervation of white adipose tissue (WAT) is the principal initiator of lipid mobilization not only in these animals, but in all mammals including humans. We present irrefutable evidence for the sympathetic nervous system (SNS) innervation of WAT with respect to neuroanatomy (including it central origins as revealed by transneuronal viral tract tracers), neurochemistry (norepinephrine turnover studies) and function (surgical and chemical denervation). A relatively unappreciated role of WAT SNS innervation also is reviewed - the control of fat cell proliferation as shown by selective chemical denervation that triggers adipocyte proliferation, although the precise mechanism by which this occurs presently is unknown. There is not, however, equally strong evidence for the parasympathetic innervation of this tissue; indeed, the data largely are negative severely questioning its existence and importance. Convincing evidence also is given for the sensory innervation of WAT (as shown by tract tracing and by markers for sensory nerves in WAT), with suggestive data supporting a possible role in conveying information on the degree of adiposity to the brain. Collectively, these data offer an additional view to the predominate one of the control of body fat stores via circulating factors that serve as efferent and afferent communicators.

INTRODUCTION

Obesity is a serious growing health concern that is associated with increases in the incidence of type II diabetes, cardiovascular disease, stroke and some cancers. Although the exact number of deaths attributable to being overweight/obese is difficult to determine precisely, it has been estimated to be as high as ~325,000 [1] or as low as ~26,000 per year [41], which translates to ranking as either the number 2 or number 7 cause of death, respectively, in adults in the United States,. Thus, the negative impact on individual health and economics, as well as, on global health and economies cannot be overestimated making obesity clearly a disease of literally and figuratively enormous proportions.

Although a vast majority of the non-human animal efforts to understand obesity is involved with factors affecting its development, we are interested in its reversal. Specifically, we have

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been studying the reversal of a naturally-occurring seasonal obesity in Siberian hamsters (*Phodpus sungorus*). Siberian hamsters become severely obese (~50 % body fat [*e.g.*, [15,53,93]]), as obese as genetically inbred strains of rats and mice bred or created for their obesity.

Changes in the photoperiod control a variety of seasonal responses including adiposity

It is critical for animals that live in temperate zones to anticipate changes in the season so that biologically important, physiological and behavioral responses occur at an optimal time-of-year. These seasonally adaptive responses are most commonly triggered by changes in the photoperiod in mammalian species (for reviews see: [13,51,91]). This photoperiod cue is received by the retina and relayed along a multisynaptic pathway to the pineal gland [49] where this photic information is transduced into an endocrine signal via the rhythmic pattern of secretion of its hormone, melatonin (MEL; 13). Thus, the duration of night is faithfully coded by the duration of nocturnal MEL secretion thereby triggering seasonal responses --long durations of circulating MEL signal the short days (SDs) of 'fall/winter' and short durations signal the long days (LDs) of 'spring/summer' [17]. The MEL receptor subtype mediating the body fat and other photoperiodic responses is the MEL_{1a} receptor (a.k.a. mt_1 receptor;[72]).

When Siberian hamsters are housed in 'summer-like' LDs (*e.g.*, 16 h light, 8 h darkness) their obesity gradually develops naturally, reaching 40-50% body fat by 2-3 mo of age (for review see: [20]). Even more remarkable is that this obesity is completely reversed when they are exposed to 'winter-like' SDs (*e.g.*, 8 h light, 16 h darkness) and this rapid loss of body fat occurs voluntarily and without, at least initially, a decrease in food intake (*e.g.*, [21,93]). In addition, unlike the other photoperiod-induced obesity models (*e.g.*,[33]), the body fat loss in SD-exposed Siberian hamsters is not uniform, with the internally-located visceral fat pads mobilizing their lipid stores first and to a greater extent than the more externally-located subcutaneous fat pads [7,8,15] making them an ideal model to study the differential deposition/mobilization of lipid from white adipose tissue (WAT).

MEL affects lipid mobilization indirectly via the sympathetic nervous system (SNS) innervation of WAT

How does melatonin affect lipolysis? MEL does not *directly* trigger lipolysis itself because incubation of isolated white adipocytes (fat cells) *in vitro*, even at 'industrial strength' non-physiological doses, does not increase lipolysis [57]. In fact, at physiological concentrations *in vitro* MEL *inhibits* the mimicry of sympathetically-mediated lipolysis by isoproterenol [102]. Perhaps MEL, a hormone itself, triggers secretion of another hormone that directly or indirectly stimulates lipolysis? We tested hormones that changed seasonally in Siberian hamsters that also either directly or indirectly affected lipolysis in these or other animals (*e.g.*, insulin, prolactin, glucocorticoids, gonadal steroids, thyroid hormones; for review see: [10,12]). This was accomplished by producing LD-like circulating concentrations of each hormone in SD-housed hamsters and *vice versa*. None of the hormones tested accounted for the photoperiodic control of body fat levels in this species (for review see: [10]). Short of testing these hormones in dual, triple or quadruple combinations, we sought other possible mechanisms for the SD-induced increases in WAT lipid mobilization; namely the possibility that MEL was stimulating epinephrine (EPI) release from the adrenal medulla that, in turn, was stimulating lipolysis. The dogma regarding lipolysis that still is pervasive is that EPI is the initiator of lipolysis. This notion is largely based on the robust lipolytic response, as measured by glycerol release, of isolated white adipocytes when incubated with physiological concentrations of EPI (*e.g.*, [73,97]). If EPI is the principle mechanism underlying lipolysis, then eliminating the sole source of the circulating EPI by removing the adrenal medulla (adrenal demedullation [ADMEDx]) should *block* lipid mobilization under conditions that promote WAT lipolysis. The inability of ADMEDx to block lipid mobilization triggered by several physiological conditions (*e.g.*, glucoprivation [58], electrical stimulation of the medial hypothalamus [90] and SD exposure in

Siberian hamsters [34]) suggests that other mechanisms are primarily responsible for lipid mobilization from WAT. We then turned to the SNS innervation of WAT, as discussed in considerable detail below, as a possible mechanism by which MEL could engage WAT to trigger lipolysis.

WAT is directly innervated by the SNS, as has been established for brown adipose tissue (BAT)

It has long been known and accepted that BAT, the primary function of which is thermogenesis, receives dense SNS innervation. Furthermore, increased SNS outflow to BAT increases thermogenesis via the uncoupling of electron transport from oxidative phosphorylation through a unique property of the mitochondrial membrane involving uncoupling protein-1 (for reviews see: [18,27]). Despite histological evidence for the SNS innervation of WAT that is over 100 years old [36], the initial reports of only SNS innervation of blood vessels within WAT strengthened the view that this innervation was solely involved in WAT blood flow [3,32,98]. This conclusion was based on the inability to detect neural fibers in the parenchymal space, likely because white adipocytes are so tightly packed. With fasting-induced increases in lipolysis, however, the cells shrink revealing catecholaminergic innervation of both the vasculature and white adipocytes (*e.g.,* [4,65,78]). We previously reported the first direct neuroanatomical bi-directional evidence of the innervation of WAT by the postganglionic neurons of the SNS using the fluorescent anterograde tracer 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) and the fluorescent retrograde tracer, FluoroGold, in Siberian hamsters [100]. Moreover, we also showed for the first time that there is a viscerotopic distribution of the postganglionic innervation of WAT, as evidenced by somewhat different patterns of labeled postganglionic cells within the sympathetic chain innervating inguinal and epididymal WAT pads (IWAT and EWAT, respectively) [100]. This relatively separate innervation pattern of WAT depots perhaps provides the neuroanatomical basis for the differential mobilization of lipid across individual WAT pads (discussed below).

The origins of the SNS innervation of WAT are known through the use of viral tract tracing methodologies

Although we defined the distribution of postganglionic SNS neurons projecting from the spinal cord to IWAT and EWAT [100], as discussed directly above, the rostral CNS origins of the innervation of the preganglionic sympathetic neurons in the spinal cord that project to these postganglionic neurons remained to be defined neuroanatomically. This was made possible by the development of a transneuronal viral tract tracer, the Bartha's K strain of the pseudorabies virus (PRV), that was used by others to trace the SNS outflow to numerous peripheral tissues before we did our studies in WAT and in BAT (*e.g.*, muscle [74], pancreas [52], heart [87], adrenal gland [89]). In brief, (for review see:[29]), neurotropic viruses, such as PRV, are taken up into neurons upon binding to viral attachment protein molecules located on the surface of neuronal membranes. Once internalized, viral components are transported to the cell soma and inserted into the nucleus where the viral progeny replicate. The newly formed progeny are then subsequently released into synaptic clefts, where they are taken up by the post-synaptic cells. This process then continues causing infections along a chain of neurons from the inoculation site, in our case WAT, to higher CNS sites. The infected neurons are visualized using standard immunocytochemistry or, because PRV is relatively easily genetically engineered, isogenic versions of the virus have been constructed to produce fluorescent reporters (*i.e.*, green [79] or red fluorescent protein [6]). Use of these fluorescent reporting PRVs eliminates immunohistochemical processing of the tissue. There are two major advantages of using viruses as transneuronal markers over some of the more traditionally used tracers: a) their ability to replicate within the neuron, and thus act as a self-amplifying cell marker across the chain of infected neurons and 2) because the transfer of the virus only is by a transynaptic mechanism, rather than by lateral spread to adjacent, but unrelated, neurons, or by a non-

synaptic mechanism [67]. Thus, PRV yields a hierarchical chain of highly target specific and functionally connected neurons labeled retrogradely from the inoculated tissue to brain (for review see: [85]).

We originally retrogradely labeled the SNS outflow from brain to WAT (both IWAT and EWAT) in laboratory rats and Siberian hamsters [5]. Briefly, neurons comprising the CNS-SNS-WAT connections were identified throughout the neural axis (for review see: [9,11]). In general, the patterns of infections among IWAT, EWAT and RWAT pads of the Siberian hamsters were more similar than different, as were the patterns of infection for IWAT pads between Siberian hamsters and laboratory rats (EWAT was not tested in rats). This is not to deny the existence of a viscerotopic sympathetic innervation of WAT at these rostral levels of the neuroaxis, but rather that careful studies using isogenic strains of the PRV, each with a unique fluorescent or other reporter, have not been done to date. Collectively, it appears that WAT receives input from CNS cell groups that are part of the general SNS outflow from brain (*i.e.*, hypothalamic paraventricular nucleus, A5 of the noradrenergic lateral tegmental system, caudal raphe region, rostral ventrolateral medulla, ventromedial medulla [88]). We found PRV labeling along the entire neuroaxis, including the brainstem: area postrema, nucleus of the solitary tract, and raphe regions (e.g., pallidus, obscurus, magnus and dorsal raphe and the raphe cap); midbrain: periaqueductal gray pontine regions and forebrain: hypothalamic arcuate, preoptic, suprachiasmatic, paraventricular and dorsomedial nuclei and thalamic paraventricular and reuniens nuclei (for a complete list see: [86]) to name a few of the more noteworthy infected areas.

The underlying neural mechanisms controlling photoperiod-induced obesity are not precisely known—The exact circuits and brain sites responsible for orchestrating the photoperiod-induced changes in body fat are not known, but recently, significant progress has been made. Given our knowledge of the SNS outflow from brain to WAT discussed above, we sought the sites of interaction of nocturnally released MEL with this outflow that could underlie the SD (MEL)-induced increase in WAT norepinephrine turnover (NETO 100; ≈; sympathetic drive) that would, in turn, promote lipid mobilization. The MEL_{1a} receptor is the functional MEL receptor subtype for photoperiodic responses in Siberian hamsters and many other photoperiodic species [66,94]. Using *in situ* hybridization developed with emulsion autoradiography, we obtained cellular resolution of neurons that expressed MEL_{1a} receptor mRNA centrally in the forebrain of Siberian hamsters with prominent MEL_{1a} receptor gene expression in the paraventricular (PVT) and reuniens (ReN) nuclei of the thalamus, and in the suprachiasmatic nucleus of the hypothalamus (SCN) and peripherally in the pars tuberalis [82-84] - results in agreement with expression pattern originally identified by others using film autoradiography [37,95]. We found co-localization of PRV-immunoreactivity with MEL_{1a} receptor mRNA in the SCN, perifornical area, periventricular fiber system, periventricular nucleus, hypothalamic paraventricular nucleus (PVN), zona incerta, subzona incerta, reuniens/xiphoid area, anterior hypothalamus and dorsomedial hypothalamic nucleus[82] (midbrain and brainstem were not sampled). Of these areas, at least the SCN appears critical in the reception of season-encoded MEL signals because we previously found that pinealectomized Siberian hamsters bearing SCN lesions do not exhibit SD-like responses, including the decrease in adiposity [14,80,81] when given MEL exogenously to mimic naturally-occurring SD MEL signals. Given that we found the colocalization of MEL_{1a} receptor mRNA expression in neurons that comprise part of the SNS outflow circuitry from brain to WAT [82] as noted above, this suggests that increases in the SNS neural drive to WAT may underlie the SD-induced increases in lipid mobilization by this species.

Siberian hamsters respond to SDs with a suite of coordinated sympathetic responses that work together to decrease body fat. That is, there is a SD-induced increase in the SNS drive to WAT evidenced as an increase in NETO [100], as discussed above. In addition, SD exposure increases

the potency/efficacy of NE-triggered lipolysis in a temporally- and fat pad-specific manner; thus, when WAT pad mass decreases most rapidly (first 5 wks of SDs), the potency (sensitivity/EC_{50}) and efficacy (maximal response asymptote) of NE-induced lipolysis is increased in isolated IWAT and EWAT adipocytes compared with their LD counterparts [24]. The SD enhancement of lipolysis was similar for NE and BRL 37344, the latter a specific β_3-adrenoceptor agonist, implying the primacy of this receptor subtype in this response [24]. In addition, we found previously that SDs up-regulate WAT β_3-adrenoceptor mRNA expression (and thus, perhaps protein), suggesting that increased β_3-adrenoceptors may mediate the SD-induced increased NE sensitivity/efficacy [35]. Therefore, there is a coordinated set of SD-triggered sympathetic responses that appear to foster the rapid decreases in body fat associated with early SD exposure (first 5 wk), collectively underlying the seemingly *effortless* shift from the obese to lean state in these animals. These decreases in body fat also occur without a significant decrease in food intake during this period [16,93], implicating, increased energy expenditure [21]. This sharply contrasts with the sympathetic and other responses associated with voluntary body fat loss in humans where there is an *opposition* to the decreases in adiposity making obesity reversal anything but *effortless* in humans.

In summary, we have determined the sequence of events from the environmental cue (*i.e.*, seasonal changes in photoperiod) to decreases in fat cell size that result in the reversal of photoperiod-induced seasonal obesity in Siberian hamsters according to the following scenario: 1) With the fall/winter increases in the duration of night (SDs), 2) there is an increase in the peak nocturnal duration of MEL secretion by the pineal gland, 3) that, in turn, increases the stimulation of MEL_{1a} receptors located on SNS outflow neurons (SCN and other areas), 4) resulting in increases in the SNS drive to WAT (increases in NETO), 5) triggering NE-mediated increases in lipolysis that are augmented by the SD-induced increases in the efficacy/potency of NE that may be due to the SD-induced increases WAT β_3-adrenoceptor number, 6) producing decreases in fat cell size that 7) ultimately result in decreases in total body fat.

The SNS innervation of WAT mediates the photoperiod/melatonin-induced increases in lipolysis in Siberian hamsters, as well as lipolysis resulting from other energetic challenges in all mammals, including humans

Increased sympathetic drive in WAT (*i.e.*, increased NETO) is increased in Siberian hamsters not only by SDs [100], but also by cold or fasting (Brito, M., Brito, N., Song, C. K., Barro, D. and Bartness, T., in preparation), as is the case in laboratory rats [44,56], all of which are positively correlated with decreases in WAT pad mass. Although these and other data are consistent with a key role of sympathetic drive in WAT lipolysis, this relation does not always hold in that 3rd ventricularly or peripherally administered leptin increases NETO in WAT, but is not always positively correlated with decreases in WAT mass [61].

The sympathetic drive across peripheral tissues or even across different WAT pads is not uniform for a given stimulus that provokes a SNS reaction. For example, glucoprivation created by injecting Siberian hamsters with the glucose analog 2-deoxy-D-glucose to block glucose utilization, increased NETO to several WAT pads (IWAT, RWAT and dorsal subcutaneous WAT [DWAT]), but not EWAT or interscapular BAT (IBAT) [19]. NETO also is not uniform across WAT pads when Siberian hamsters are exposed to SDs with the more internally located pads (EWAT and RWAT) showing earlier and greater sympathetic drives than IWAT [100], or across WAT pads when leptin is given centrally or peripherally to laboratory rats [61]. In other situations, such as cold exposure, the sympathetic drive is increased to both WAT and IBAT (50% NETO increases in IBAT and 30% NETO increases in EWAT and RWAT; [44]). The underlying mechanism for the differential trafficking of sympathetic drive across peripheral tissues remains to be determined.

Are there common neurons in SNS outflow circuits to WAT and to BAT?

As noted above, there are many examples of differential sympathetic stimulation of target peripheral tissues both between types of tissues (WAT vs BAT), as well as within a type of tissue (different WAT pads). There are conditions, however, where multiple tissues are simultaneously sympathetically stimulated - one such example is cold exposure. In cold exposure the SNS drive to WAT is increased [44] and also is increased to BAT (*e.g.*,[39,44,99]). Cold-induced increases in the sympathetic drive to BAT are expected because this typically increases thermogenesis and the increases in the sympathetic drive to WAT could provide fuel for the heat production by BAT and other tissues. We used two reporter recombinants (PRV614 [generously supplied by Bruce Banfield, Colorado State Univ] and PRV152 and generously supplied by Lynn Enquist, Princeton University) and injected PRV152 into IWAT and PRV614 into the ipsilateral IBAT. In this preliminary study, we found double-labeled (PRV 614 + PRV 152) neurons in several CNS regions including the hypothalamic PVN supporting the notion of shared SNS outflow to these two types of adipose tissues (85 and N. Brito, M. Brito, C. K. Song, M. Wesley, J. Randall, B. Banfield and T. Bartness, in preparation).

The neurochemical phenotypes of the SNS outflow circuits to WAT are largely unknown, but a majority of neurons making up the components in this circuit across the neuroaxis express melanocortin receptors—Although we have demonstrated co-localizations of PRV-infected neurons with arginine vasopressin [75], tyrosine hydroxylase [75], oxytocin [75], acetylcholine transferase [45], MEL_{1a} receptors [82]) we are only beginning to understand the complex neurochemical phenotypes of the neurons comprising the sympathetic outflow circuits to WAT. In terms of the latter, the melanocortins have been heavily implicated in the control of food intake and energy expenditure (for review see: [38, 92]). In addition, the and melanocortin-4 receptors [MC4-Rs] also have been implicated in SNS-mediated WAT lipolysis [64], in that central application of theMC3/4-R agonist, melanotan-II to laboratory rats results in decreases in food intake and body fat and, moreover, the decreases in body fat are greater than can be accounted for by the decreases in food intake, as revealed by pair feeding [64]. These results suggest that stimulation of MC4-Rs (the melanocortin subtype receptor most noted for effects on energy balance, *e.g.*, [26,38]), increase WAT lipid mobilization, perhaps via the SNS innervation of WAT, as well as increases in thermogenesis, perhaps by BAT and via its SNS innervation. This begs the question as to whether sympathetic outflow neurons posses MC4-Rs? We tested this notion by first labeling the SNS outflow to WAT using PRV in Siberian hamsters and then processing the brains for MC4-R mRNA using *in situ* hybridization [86]. We found extensive co-localization of MC4-R mRNA with PRV-labeled SNS outflow neurons across the neural axis with the percentage of double-labeled cells at least ~60% for most brain areas [86]. Large numbers of PRV + MC4-R neurons were found in the hypothalamic paraventricular nuclei, preoptic area, bed nucleus of the stria terminalis and amygdala in the forebrain, periaqueductal gray in the midbrain and the nucleus of the solitary tract, lateral paragigantocellular nucleus, lateral reticular area, rostroventrolateral medulla and anterior gigantocellular nucleus in the brainstem, to name only a few of the more predominant sites of co-localization. This is the most extensive co-localization we have seen to date in our studies or those of others for PRV and a variety of neurochemicals (for brief review, see Discussion in [75]) and suggests that MC4-Rs play a prominent role in the modulation of SNS outflow neurons to WAT either through stimulation by the endogenous melanocortin agonist α-melanocyte-stimulating hormone and/or through inhibition by the naturally-occurring MC3/4-R reverse agonist, agouti-related protein (AgRP; 60).

Functional evidence for the role of the sympathetic innervation of WAT in lipid mobilization—Although the neuroanatomical and neurochemical data presented above build a reasonable case for the role of the sympathetic innervation of WAT in lipid mobilization, functional evidence is critical to establish such a role beyond doubt. The necessity of obtaining

such functional evidence is exemplified by the initial, seemingly credible role of adrenal medullary EPI in WAT lipolysis that was severely undermined by inability of ADMEDx (and thus no circulating EPI) to block lipid mobilization under a variety of conditions (see above). Functional studies of the SNS innervation of WAT have taken the advantage of the unilateral innervation of paired WAT pads. This 'unilateral denervation model' involves denervating one of a pair of WAT pads with its contralateral mate serving as a within-animal, neurally intact control that receives sham denervation. The beauty of this model is that one of the bilateral WAT pads is denervated and the other is not; thus, all other characteristics of the animal are held constant such as all circulating factors, the age of the animal, its nutritional status, energy expenditure and food intake. Using this model, when WAT is surgically or neurochemically denervated, then lipid mobilization from this pad is profoundly compromised compared with its neurally intact contralateral mate across a number of treatments and species such as fasting of laboratory rats, cats, rabbits, and dogs [23,25,28,30,96], estradiol treatment of ovariectomized rats [50], or SD exposure in Siberian hamsters [101]. Such local surgical denervation offers more neuroanatomical specificity than does global sympathectomy using guanethidine [63] or 6-hydroxy-dopamine (6OHDA; 71), however, it is not neuroanatomically selective, because both autonomic *(i.e.*, sympathetic; see below for a discussion on parasympathetic nervous system [PSNS] innervation of WAT [22,45]) and sensory nerves are severed. Indeed, surgical denervation significantly decreases both tyrosine hydroxylase-immunoreactivity (a sympathetic nerve marker) and calcitonin gene-related peptide (CGRP)-immunoreactivity (a sensory nerve maker), indicating reduced sympathetic and sensory innervations, respectively [42,76,77]. Selective SNS denervation using *locally-injected* guanethidine, however, blocks SD-induced lipid mobilization in Siberian hamsters [34]. Thus, these and other data provide unquestionable evidence that increased sympathetic neural drive to WAT is the primary stimulus triggering lipid mobilization.

Does WAT have PSNS innervation?—Most tissues are dually innervated by both arms of the autonomic nervous system - the SNS and PSNS - that in general, functionally oppose one another. One report suggests that WAT also has PSNS innervation [48] in addition to its well documented SNS innervation. A thorough discussion of these findings recently has been published [22,45], therefore, this issue only will be briefly summarized here. In the study by Kreier *et al* [48], selective surgical denervation of the sympathetic nerves innervating WAT, that would ostensibly spare its PSNS innervation, was followed by PRV injections into the sympathetically denervated WAT to label the putative PSNS outflow to WAT. This resulted in extensive bilateral infection of the dorsal motor nucleus of the vagus (DMV) among other areas. Because the PSNS innervation of WAT was supposedly spared, this infection only could have occurred because of retrograde labeling of the multisynaptic PSNS circuit from brain to WAT [48]. We find these results quite remarkable. First, bilateral DMV infection resulting from unilateral PRV injection into WAT only adds to the confusion because vagal DMV efferents innervate peripheral tissues unilaterally in rodents (*e.g.*, [59,62]). By contrast, there is accumulating evidence arguing *against* PSNS innervation of WAT. For example, there is: a) no identification of markers of WAT PSNS nerves at the level of the WAT pad or elsewhere (2 and see below), b) no identification of PSNS ganglia in or near the WAT pads [22] and c) as noted above, the DMV innervates most peripheral tissues unilaterally in rodents [59,62]. For these and other reasons outlined recently [22,45], we sought PSNS nerve markers in WAT [45] using three types of animals: a standard mouse strain (C57BL mice), a genetically obese mouse strain (*ob/ob* mice) and a standard laboratory rat strain (Sprague-Dawley rats). We examined three WAT pads: a) IWAT, RWAT and EWAT and tested for three neurochemical markers of PSNS shown in other tissues: a) vesicular acetylcholine transporter (*e.g.*, skeletal muscle, salivary glands, intestine and heart), b) vasoactive intestinal peptide (*e.g.*, intestine) and c) neuronal nitric oxide synthase (*e.g.*, gastric fundus). Thus, we used three animal models, three

WAT pads and three proven markers of parasympathetic nerves, but the results pointed to one conclusion ---no PSNS innervation of WAT [45].

We tested the SNS denervation of WAT for ourselves in an attempt to spare the PSNS innervation, but we opted for a local neurotoxin approach to achieve this destruction [45]. Specifically, we injected the catecholaminergic neurotoxin 6-hydroxy-dopamine (6OHDA) locally in one IWAT pad of Siberian hamsters, whereas its contralateral mate received injections of the vehicle (*i.e.* we used the unilateral denervation model discussed above). 6OHDA applied locally produces a selective sympathetic denervation as indicated by significantly decreased NE content and tyrosine hydroxylase-immunoreactivity with no effect on calcitonin gene-related peptide-immunoreactivity, the sensory nerve marker [42,77]. Subsequent PRV injection several days later into 6-OHDA denervated WAT resulted in no brain, spinal cord or sympathetic chain infections suggesting no PSNS innervation, whereas vehicle-injected WAT subsequently inoculated with PRV had typical viral infection patterns across the neuroaxis. Collectively, these data suggest that either the extent of the PSNS innervation is so insignificant that looking for it at the level of the fat pad is equivalent to looking for a needle in a haystack and therefore questions its biological importance or its existence [45].

Does WAT have sensory innervation?—Sensory innervation of tissues is the rule, not the exception, so it should not be surprising that there is sensory innervation of WAT (for reviews see: [9,11]). WAT afferents have been demonstrated by several methods. The sensory innervation of WAT was initially suggested by the identification of substance P in WAT [43]. Substance P and calcitonin gene-related peptide are contained within, and released from, sensory neurons and thus considered markers of sensory innervation [46]. We demonstrated the presence of calcitonin gene-related peptide in nerves at the level of WAT pads in Siberian hamsters [42,76,77]. Sensory innervation of WAT was first directly shown neuroanatomically by applying an anterograde tract tracer, True Blue, to WAT that resulted in labeling of neurons in the dorsal root ganglia of laboratory rats [40]. This labeling of pseudounipolar sensory neurons in the dorsal root ganglion using tract tracing is the *sine quo non* of identifying neural connection, however, this approach cannot tell us the sensory projections to more rostral areas of the neuroaxis such as the forebrain, midbrain brainstem and spinal cord. To address this issue, we used the viral tract tracing approach of Rinaman and Schwartz [68] who defined the sensory nerve projections from the stomach to the CNS using the H129 strain of herpes simplex virus-1 [103]. Unlike PRV, H129 only initially travels retrogradely from the site of inoculation in the periphery, in our case from IWAT to the soma of the pseudounipolar sensory neurons in the dorsal root ganglion, at which point it replicates and then only travels anterogradely such that the sensory afferent projections to the brain become infected. Using standard immunohistochemistry, we found H129-immunoreactive cells at all levels of the neuroaxis, including both the nodose ganglia (visceral afferents) as well as the dorsal horn of the spinal cord (spinal afferents) and in many of the classic autonomic output areas in the brainstem/pons and midbrain including the rostroventrolateral medulla, midbrain areas including the lateral periaqueductal gray, lateral parabrachial nucleus, subcoeruleus, and forebrain areas including the lateral hypothalamus, subzona incerta, periventricular area, posterior hypothalamus, preoptic area, hypothalamic paraventricular nucleus and sub-paraventricular area, bed nucleus of the stria terminalis, and lateral septum, to name a few. Although some of the rostral brain sites that comprise the sympathetic outflow circuit from brain to WAT also showed infected neurons after H129 inoculation of IWAT, there was not a complete overlap (C. K. Song and T. Bartness, in preparation).

What are the sensory nerves monitoring?—Quite frankly, at this time we do not know. We hypothesize that the sensory information may provide the brain with some assessment of body fat levels. For example, WAT sensory nerve endings could respond to the products of

lipolysis (glycerol, free fatty acids) or other factors that might correlate with the lipid content of the adipocytes, such as the cytokine leptin [104]. We demonstrated that WAT sensory nerves could be experimentally manipulated by ablating them with local injections of the unmyelinated sensory nerve neurotoxin capsaicin [76]. Capsaicin is the pungent part of red chili peppers and typically is given systemically to produce global sensory denervations (*e.g.,* [31, 69], or locally for restricted sensory denervations, for example of the gastrointestinal system [47]). Systemic capsaicin, however, not only produces a general peripheral sensory denervates of the periphery, but also kills neurons in several brain areas [70]. The denervation is selective, not affecting tyrosine hydroxylase-immunoreactivity indicative of SNS innervation, but largely eliminating or at least significantly decreasing calcitonin gene-related peptide-immunoreactivity, the latter a marker of sensory innervation [77].

We attempted to achieve an initial understanding of the function of sensory nerves in WAT as a possible mediator of lipectomy-induced body fat compensation (for review see: [55]). When WAT is surgically removed, the remaining unexcised WAT pads increase in mass in an apparent attempt to compensate for the lipectomy-induced lipid loss. This is a robust response, seen in a variety of species including Siberian and Syrian hamsters, ground squirrels, laboratory rats and mice, sheep, with some suggestive data from humans, as well (for review see: [55]). The signal that triggers this compensation is unknown, but could involve disruption of WAT sensory innervation that accompanies lipectomy. Therefore, we compared local and selective sensory denervation, accomplished by microinjecting capsaicin bilaterally into EWAT of Siberian hamsters, with controls that received vehicle injections, to the more traditional bilateral EWAT lipectomy (EWATx) or sham lipectomy [76]. As we saw previously (*e.g.,* [53, 54]), EWATx triggered a significant increase in the masses of RWAT and IWAT and similarly, capsaicin did the same, even though there was no actual lipid deficit rather only selective sensory denervation, as verified by significantly decreased calcitonin gene-related peptide-immunoreactivity, but not tyrosine hydroxylase-immunoreactivity [76]. Moreover, this compensatory increase in the masses of the unexcised WAT pads collectively closely approximated the mass of the paired EWAT pads had they been removed [76]. These data suggest that the destruction of sensory nerves that accompanies surgical lipectomy is the trigger for the compensatory increases in WAT mass in the non-excised WAT pads and further suggests the possibility that the size of lipid stores may be relayed to the brain via this sensory nerve conduit to inform the brain of adiposity levels [76].

Conclusions—This review presents strong neuroanatomical, neurochemical and functional evidence for WAT SNS innervation, with no solid neuroanatomical evidence for PSNS innervation of WAT at the level of the fat pad. Strong neuroanatomical evidence also was presented for WAT sensory innervation and one function of those nerves may be to sense body fat levels. The apparent trend in biomedical, obesity-related research is to focus on circulating factors, such as leptin, that under some circumstances seem to provide the brain with information on body fat levels, as well as on adrenal medullary EPI as a means of mobilizing lipid. The data reviewed here, however, are suggestive of an alternative hypothesis for the control of body fat than by circulating factors - efferent and afferent innervations of WAT. The evidence that the SNS innervation of WAT is the principal initiator of lipid mobilization is incontrovertible. It is premature to ascribe importance for the sensory innervation of WAT as a means of conveying lipid-related information to the CNS from WAT, but the data to date are undeniable for the existence of this innervation and the functional data are intriguing in terms of a possible role in sensing lipid levels in WAT.

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