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Sensory and sympathetic nervous system control of white adipose tissue lipolysis

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Summary

Circulating factors are typically invoked to explain bidirectional communication between the CNS and white adipose tissue (WAT). Thus, initiation of lipolysis has been relegated primarily to adrenal medullary secreted catecholamines and the inhibition of lipolysis primarily to pancreatic insulin, whereas signals of body fat levels to the brain have been ascribed to adipokines such as leptin. By contrast, evidence is given for bidirectional communication between brain and WAT occurring via the sympathetic nervous system (SNS) and sensory innervation of this tissue. Using retrograde transneuronal viral tract tracers, the SNS outflow from brain to WAT has been defined. Functionally, sympathetic denervation of WAT blocks lipolysis to a variety of lipolytic stimuli. Using anterograde transneuronal viral tract tracers, the sensory input from WAT to brain has been defined. Functionally, these WAT sensory nerves respond electrophysiologically to increases in WAT SNS drive suggesting a possible neural negative feedback loop to regulate lipolysis.

Keywords

obesity; (Siberian hamster); denervation; norepinephrine; calcitonin gene-related peptide

Introduction to the Sympathetic and Sensory Innervation of White Adipose Tissue

Historically, the control of white adipose tissue (WAT) lipolysis has focused on circulating factors. Specifically, the adrenal medullary catecholamines epinephrine (EPI), and to a lesser degree norepinephrine (NE), have been acknowledged as the principal initiators of lipolysis, whereas pancreatic insulin is well-acknowledged as a potent inhibitor of this process (Froesch et al. 1965; Goodridge and Ball 1965; Prigge and Grande 1971). Similarly, the focus of the communication between WAT and the brain has centered on factors secreted from this tissue

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including leptin and adiponectin, as well as other 'adipokines' [for review see: (Trayhurn and Bing 2006)]. This review will instead have a neural theme -- the bidirectional communication between brain and WAT occurring via the sympathetic nervous system (SNS) and sensory innervation of this tissue [for review see: (Bartness et al. 2005; Bartness and Bamshad 1998; Bartness and Song 2007)]. This neural theme stems from our ability to trace efferent and afferent circuits to and from WAT, respectively, with transneuronal viral tract tracers, as we first did for its SNS innervation using pseudorabies virus [PRV; (Bamshad et al. 1998)] and more recently for its sensory innervation using the H129 strain of herpes simplex virus-1 (Song et al. 2009a). We will begin by briefly examining the background studies behind the notion that the SNS innervation of WAT is the principal initiator of lipolysis. Next, we will evaluate the current status of our knowledge regarding the sensory innervation of WAT. Finally, we will speculate on how the sensory and sympathetic innervation of this tissue may interact to at least partially control lipolysis and more generally, body fat levels.

A Seasonal Model of Obesity Reversal Led to the Realization of the Importance of the Sympathetic Innervation of WAT for Lipid Mobilization

The impetus for testing the role of the SNS innervation of WAT in lipolysis was our study of the reversal of a naturally-occurring seasonal obesity in Siberian hamsters [*Phodopus sungorus*; for review see: (Bartness et al. 2002; Bartness and Wade 1985; Morgan et al. 2003)]. Their impressive obesity (~50% of body mass as body fat) peaks in the long days (LDs) of summer and is reversed to a more modest level of adiposity (~20% of body mass as body fat) in the short days (SDs) of winter in the field (Weiner 1987). This natural reversal of their seasonal obesity can be mimicked in the laboratory by changing only the photoperiod from LDs to SDs, while holding all other environmental factors constant. [(Bowers et al. 2005; Wade and Bartness 1984); for review see: (Bartness, Demas, & Song 2002; Bartness & Wade 1985)]. In Siberian hamsters and other species, the daylength (photoperiod) cue is translated into a neuroendocrine signal via the duration of the nocturnal secretion of melatonin (MEL) from the pineal gland, secreted in direct proportion to the length of the dark period [for review see: (Bartness et al. 1993; Bartness and Goldman 1989; Goldman 2001)]. The MEL_{1a} receptor subtype mediates photoperiodic responses [*e.g.*, (Roca et al. 1996)]. MEL itself, however, does not *directly* trigger WAT lipolysis, as incubation of isolated white adipocytes with clearly supraphysiological doses of MEL does not increase lipolysis (Ng and Wong 1986). We therefore sought to identify circulating factors that changed seasonally in these animals that would either directly or indirectly affect lipolysis (*e.g.*, insulin, thyroid hormones, prolactin, gonadal steroids, glucocorticoids), but none could account for this obesity reversal [for review see: (Bartness, Demas, & Song 2002)].

At that time, EPI was considered by most to be the principal initiator of lipolysis because of its ability to robustly stimulate lipolysis (glycerol release) in isolated white adipocytes [*e.g.*, (Bukowiecki et al. 1980; Prigge & Grande 1971; Rochon and Bukowiecki 1990; White and Engel 1958)]. We therefore tested whether changes in the secretion of adrenal medullary catecholamines (NE, EPI) triggered SD (MEL)-induced increases in lipid mobilization. Adrenal demedullation, however, did not block the reduction in body fat caused by transfer from LDs to SDs (Demas and Bartness 2001). This lack of a significant role of adrenal medullary catecholamines in SD-induced lipid mobilization in Siberian hamsters is supported by similar results from studies of adrenal demedullated laboratory rats following a variety of lipolytic challenges [*e.g.*, glucoprivation (Nishizawa and Bray 1978), electrical stimulation of the medial hypothalamus (Takahashi and Shimazu 1981)]. Given that we previously tested likely humoral candidates for their role in SD-induced lipid mobilization and found none could account for the increases in lipolysis, as noted above [for review see: (Bartness, Demas, & Song 2002)], the SNS innervation of WAT appeared to be the only apparent remaining untested mechanism underlying this obesity reversal. This was possible because MEL readily crosses

the blood brain barrier (Vitte et al. 1988) and MEL receptors have been localized on brain neurons [for review see: (Bittman 1992; Reppert 1997)]. Sympathetic drive-stimulated lipolysis would most easily explain the non-uniform mobilization of lipid in SDs, whereby internal WAT pads [*i.e.*, epididymal WAT (EWAT), retroperitoneal WAT (RWAT)] are reduced to a greater extent and before that of subcutaneous WAT [*i.e.*, inguinal white adipose tissue (IWAT)]; (Bartness et al. 1989; Bartness 1995; Bartness 1996)]. Humans also exhibit differential lipid mobilization between visceral and subcutaneous WAT with exercise, dieting/ and fasting [*e.g.*, (Bjorntorp et al. 1975; Krotkiewski 1988)]. Therefore, the possible mediation of lipolysis via increases in the sympathetic drive to WAT hinged on strong evidence of the SNS innervation of this tissue.

White Adipose Tissue is Innervated by the Sympathetic Nervous System

Before the first direct evidence (*i.e.*, tract tracing of the neurons innervating this tissue) of the SNS of WAT (Youngstrom and Bartness 1995), there were strong suggestions of such innervation determined at the level of the adipose tissue, albeit not without some confusion. It was initially reported that small diameter fibers make apparent contact with BAT, but not WAT adipocytes (Wirsén 1965). Using histofluorescence, the sympathetic innervation of WAT vasculature then became clear (Ballantyne and Raftery 1974; Daniel and Derry 1969), and moreover, using the same methodology, both vascular and parenchyma catecholaminergic innervation was identified in dogs (Ballard et al. 1974) and laboratory rats (Diculescu and Stoica 1970; Rebuffe-Scrive 1991; Slavin and Ballard 1978). Most importantly, parenchymal innervation of WAT adipocytes was observed at the electron microscopic level, albeit at relatively low levels of innervation [~2-3% of adipocytes (Slavin & Ballard 1978)]. Although apparently sparse, these adipose endings were of the *en passant* variety, where the release of NE at each 'terminal' would affect many surrounding cells (Slavin & Ballard 1978).

Traditional single neuron tract tracers [by contrast to multi-synaptically traveling tract tracers (see below)] revealed direct postganglionic SNS innervation of WAT (Youngstrom & Bartness 1995). These data, however, did not address the origins of the sympathetic outflow to WAT from the brain. Therefore, using viral transynaptic tract tracing methodology [for review see: (Song et al. 2005a)], we were able to define the origins of the SNS outflow from brain to WAT for the first time in any species (Bamshad, Aoki, Adkison, Warren, & Bartness 1998) using pseudorabies virus (PRV). PRV is a retrograde viral tract tracer [for review see: (Ekstrand et al. 2008)] that has been used to identify the central premotor sympathetic neurons that ultimately project to their peripheral targets for a variety of tissues. In brief, when PRV is injected into a target tissue, the virions are transported into the neuron terminals where they travel to the neuronal soma, replicate and then leave the infected neurons via their dendrites to be transferred only to other neurons making synaptic contact with the infected neurons. This process continues at each successive circuit relay site, resulting in the labeling of a hierarchical chain of functionally connected neurons extending from its termination to its origins [for review see: (Card and Enquist 1999)]. The infected neurons are identified using either standard immunohistochemistry or fluorescent microscopy for PRV that has been genetically engineered to produce fluorescent reporters (Banfield et al. 2003; Smith et al. 2000).

Using PRV tract tracing methodology, we retrogradely labeled the SNS outflow from brain to IWAT and EWAT in laboratory rats, as well as to IWAT, EWAT and retroperitoneal WAT (RWAT) in Siberian hamsters (Bamshad, Aoki, Adkison, Warren, & Bartness 1998; Bowers et al. 2004). Dozens of sites across the neuroaxis were revealed as part of the CNS-SNS-WAT circuitry (Bamshad, Aoki, Adkison, Warren, & Bartness 1998) and we subsequently did similar studies to define the CNS-SNS- brown adipose tissue (BAT) circuitry as well (Bamshad et al. 1999). The CNS infection patterns resulting from PRV injections into these various WAT pads were similar in that the same brain nuclei/regions were labeled regardless of which fat depot

was injected with PRV; however, some brain sites had greater degrees of infection than others suggesting the possibility of differential innervation (Bamshad, Aoki, Adkison, Warren, & Bartness 1998; Bowers, Festuccia, Song, Shi, Migliorini, & Bartness 2004). Others have reported some indication of 'viscerotropic' SNS innervation of WAT using two strains of PRV, each of which has a unique reporter, by injecting them into separate fat depots (Kreier et al. 2006); however quantification of these innervations was not performed.

White Adipose Tissue Does Not Appear to be Innervated by the Parasympathetic Nervous System

Many tissues are innervated by both the SNS and the parasympathetic nervous system [PSNS; a notable exception is peripheral blood vessels except for some facial blood vessels (Izumi 1995; Ruskell 1971)] with the functional consequence of each producing opposing physiological responses [for review see: (Loewy and Spyer 1990)]. Thus, although there is cholinergic *sympathetic* innervation of skeletal muscle vasculature in some species, blood vessels of the skeletal musculature in laboratory rats and mice lack this type of SNS innervation and instead have noradrenergic innervation as evidenced by abundant tyrosine hydroxylase (rate limiting enzyme for catecholamine synthesis) immunoreactivity as well as that for neuropeptide Y, the latter often found co-localized in sympathetic nerves. No immunoreactivity for classic cholinergic markers such as vasoactive intestinal peptide, vesicular acetylcholine transporter or acetylcholinesterase also are present in laboratory rat or mouse skeletal muscle vasculature (Guidry and Landis 2000). Therefore, these data and other data give no evidence for PSNS innervation of peripheral blood vessels, at least in skeletal muscle, although unsubstantiated claims of PSNS innervation exist [*e.g.*, (Schafer et al. 1998)].

Similarly, unsubstantiated claims of PSNS innervation of WAT exist (Kreier et al. 2002). This claim has been severely challenged (Berthoud et al. 2006; Berthoud et al. 2007; Giordano et al. 2006; Giordano et al. 2007) based on numerous criticisms of their viral tract tracing procedures as well as other key missing details that would support PSNS innervation. For example, several key aspects of autonomic nervous system innervation of tissues have not been identified for the proposed PSNS innervation of WAT such as the presence of parasympathetic ganglia, the presence of biochemical indicators of PSNS innervation (Ballantyne 1968) and the presence of neurochemical markers of PSNS innervation. In an analogous approach to the one used to test the presence of parasympathetic innervation of skeletal muscle blood vessels discussed above (Guidry & Landis 2000), we tested for three proven immunohistochemical markers of PSNS innervation (vesicular acetylcholine transporter, vasoactive intestinal peptide, neuronal nitric oxide synthase) in three WAT pads (IWAT, RWAT, EWAT) from three species (C57BL mice, *ob/ob* mice and Sprague-Dawley rats) and did not find a single case of labeling for any of these established PSNS markers in any WAT pad of any species (Giordano, Song, Bowers, Ehlen, Frontini, Cinti, & Bartness 2006). Moreover, in an attempt to functionally label the proposed PSNS innervation of WAT, we first produced a local and selective chemical sympathetic denervation using intra-WAT injections of the catecholamine neurotoxin, 6-hydroxy-dopamine to eliminate the SNS innervation as we had done previously (Foster and Bartness 2006; Giordano, Song, Bowers, Ehlen, Frontini, Cinti, & Bartness 2006; Rooks et al. 2005). Next we injected PRV into the SNS denervated tissue to label the proposed PSNS outflow from brain to WAT that should have been spared (Giordano, Song, Bowers, Ehlen, Frontini, Cinti, & Bartness 2006). There were, however, no infections in the sympathetic chain, spinal cord or brain in any of the animals, but normal viral SNS infection patterns in each of these neuroaxis parts in vehicle-injected WAT inoculated with PRV suggesting a complete lack of significant WAT PSNS innervation and the presence of only WAT SNS innervation (Giordano, Song, Bowers, Ehlen, Frontini, Cinti, & Bartness 2006). In addition, to provide more conclusive evidence for or against the presence of a substantial innervation of WAT other than the sympathetic supply, WAT nerves were double-stained by immunofluorescence using

antibodies against PGP9.5, a pan-neuronal marker (Thompson et al. 1983; Wilson et al. 2005), and tyrosine hydroxylase (TH), the specific marker for sympathetic noradrenergic nerves (Flatmark 2000). Adult or growing *ob/ob* obese mice had only very few fibers innervating blood vessels and the WAT parenchyma that were not noradrenergic (Giordano, Song, Bowers, Ehlen, Frontini, Cinti, & Bartness 2006) and likely sensory (see below). Thus, if PSNS innervation of WAT exists, it uses unidentified and unique PSNS neurochemicals and/or it is extremely sparse suggesting very limited physiological function, despite claims to the contrary [*e.g.*, (Kreier, Fliers, Voshol, Van Eden, Havekes, Kalsbeek, Van Heijningen, Sluiter, Mettenleiter, Romijn, Sauerwein, & Buijs 2002; Kreier and Buijs 2007)].

Melatonin Receptors Are Expressed by Sympathetic Outflow Neurons from Brain to White Adipose Tissue

To identify how SNS outflow to WAT could stimulate SD reductions in body fat via lipid mobilization in Siberian hamsters, we tested whether MEL_{1a} receptor mRNA was co-localized with the SNS outflow forebrain neurons as revealed by PRV, using emulsion autoradiographic *in situ* hybridization and immunohistochemistry, respectively (Song and Bartness 2001). MEL_{1a} mRNA was extensively co-localized with SNS outflow neurons in several forebrain sites (Song & Bartness 2001), but we cannot exclude midbrain and brainstem contributions because similar experiments have not yet been done for these brain regions. These data demonstrating the neuroanatomical reality of MEL_{1a} receptors on SNS outflow neurons to WAT, together with the SD-induced increases in sympathetic drive to WAT [as evidenced by increases in NE turnover (NETO; a neurochemical measure of sympathetic drive; (Youngstrom & Bartness 1995)], suggest the following scenario. The increases in nightlength associated with SDs consequently produce increases in the duration of pineal MEL secretion from the pineal (Hastings et al. 1991). This results in increases in the stimulation of MEL_{1a} receptors on SNS outflow neurons to WAT triggering increases in the sympathetic drive to this tissue. This would, in turn, initiate lipolysis thereby driving the SD-induced increases in lipid mobilization seen in this species. Indeed, unlike the inability of adrenal demedullation to block WAT lipolysis in SD-exposed hamsters (Demas & Bartness 2001), SNS denervation of WAT blocks this lipid mobilization (Demas & Bartness 2001; Youngstrom and Bartness 1998) and attenuates food deprivation induced lipolysis in laboratory rats, cats, rabbits, and dogs (Beznak and Hasch 1937; Bray and Nishizawa 1978; Cantu and Goodman 1967; Clement 1950; Wertheimer 1926), as well as estradiol-induced lipolysis in ovariectomized rats (Lazzarini and Wade 1991). A similar blockade of lipid mobilization in humans was seen incidentally nearly one hundred years ago when a hemiplegic patient also presented with cancer cachexia resulting in a mobilization of lipid only from the neurally intact leg (Mansfeld and Muller 1913). This blockade of lipid mobilization by sympathetic denervation of WAT demonstrates incontrovertible evidence of the necessity of this innervation for lipolysis engendered by these stimuli (and likely others, but see below for an exception).

Complementary data come from results of electrical stimulation studies. In *ex vivo* preparations of laboratory rat EWAT-sympathetic nerve explants, electrical stimulation of the attached nerves markedly increases the free fatty acid (FFA) concentration of the medium suggesting lipolysis (Correll 1963). This effect is blocked by preincubation with a β -adrenoceptor antagonist prior to electrical stimulation (Weiss and Maickel 1965). Similarly, in an analogous *in vivo* preparation in humans, (Dodt et al. 1999; Dodt et al. 2000; Dodt et al. 2003), intraneural stimulation of the lateral femoral cutaneous nerve that innervates subcutaneous WAT increases lipolysis (glycerol concentrations in the perfusate) *in situ* as measured by microdialysis (Dodt, Lonnroth, Fehm, & Elam 1999; Dodt, Lonnroth, Fehm, & Elam 2000). Thus, both denervation and stimulation studies strongly support an important role for sympathetic innervation in initiating lipolysis in WAT in human and rodents.

Sympathetic Drive to White Adipose Tissue is Not Uniform

Importantly, SNS drive to body fat is not homogeneous. Using a neurochemical measure of NETO, we have found what we term 'signature NETO patterns' that demonstrate differential SNS drive across WAT pads and interscapular BAT (IBAT). Specifically, there appears to be a somewhat unique set of differential sympathetic drives to different WAT pads and/or IBAT for each lipolytic stimulus tested to date. For example, the more internally located WAT pads (RWAT, EWAT) show the greatest increases in NETO compared with more externally located WAT pads (IWAT) in SD-exposed Siberian hamsters (Youngstrom & Bartness 1995)]. Food deprivation provokes significant increases in IWAT and EWAT NETO, but not in RWAT, DWAT or IBAT (Brito et al. 2008). By contrast, cold exposure increases NETO to IWAT, EWAT, RWAT, IBAT but not DWAT (Brito, Brito, & Bartness 2008). The glucoprivic stimulus, 2-deoxy-d-glucose, increases NETO to IWAT, RWAT and DWAT, but not to EWAT or IBAT (Brito, Brito, & Bartness 2008). Finally, central melanocortin receptor agonism caused by a single 3rd ventricular melanotan II (MTII) injection increases NETO to IWAT, and DWAT, but not EWAT or RWAT (Brito et al. 2007). Thus, differential WAT NETO seems to be the norm and is relatively fat pad-specific for various lipolytic stimuli. The mechanisms underlying how sympathetic drives are trafficked across peripheral tissues in general, a process essential for maintaining homeostasis, remain an important, yet unsolved biological mystery [for review see: (Morrison 2001)]. A potentially critical key to unlocking the door to enlightenment on this issue may be the role of the sensory innervation of WAT discussed in detail below.

Adrenoceptor Subtype, Number and Affinity Determine Lipid Mobilization From White Adipose Tissue

SNS-triggered lipolysis relies on the number, affinity and type of adrenergic receptors (adrenoceptors) on the fat cell membrane [for review see: (Collins et al. 2004;Langin 2006)]. The studies of Lafontan, Langin and associates (Carpene et al. 1993;Lafontan et al. 1979;Langin et al. 1991;Mauriege et al. 1988) have clearly delineated the function and importance of the white adipocyte adrenoceptors for catecholamine-associated lipolysis [for review see: (Lafontan et al. 1985;Lafontan and Berlan 1995)]. An unambiguous and well-substantiated outcome of their work is the notion that the balance between lipolysis-promoting β -adrenoceptor (β -AR) subtype 1-3 (β -AR₁₋₃) activation and lipolysis-inhibiting α_2 -AR activation dictates the degree of lipolytic status of the adipocyte, all other factors being equal (Collins et al. 2007;Lafontan et al. 1995;Lafontan and Berlan 1993). Thus, when β -AR activation predominates [in rodents, primarily the β_3 -ARs (Collins, Migliorini, & Bartness 2007)], lipolysis is stimulated and, conversely, when α_2 -AR activation predominates, lipolysis is inhibited (Lafontan 1994;Lafontan & Berlan 1995). Participation of each of the three β -adrenoceptor subtypes in lipolysis varies with several factors, including the WAT depot, animal species and its sex, age or degree of obesity [*e.g.*, (Carpene et al. 1998)]. β_{1-3} AR agonists activate the GTP-binding protein G_s, triggering increases in cAMP production by adenylyl cyclase that, in turn, stimulates protein kinase A (PKA). PKA phosphorylates two key players in lipolysis: hormone-sensitive lipase (HSL) and perilipin A [pHSL and pPerilipin A, respectively (Arner 2005;Carmen and Victor 2006;Langin 2006)] ultimately resulting in lipolysis. Conversely, α_2 AR stimulation decreases cAMP due to inhibition of adenylyl cyclase; thus, PKA is not stimulated and HSL and perilipin A are not phosphorylated (Arner 2005;Carmen & Victor 2006;Langin 2006).

A Series of Lipases Work Together to Fully Hydrolyze Triacylglycerol in White Adipose Tissue

Until recently, HSL was thought to be the major regulator of lipolysis. Adipose triglyceride lipase [ATGL; a.k.a. desnutrin; (Haemmerle et al. 2006; Villena et al. 2004)] also is involved in lipolysis, where it can work in conjunction with HSL and monoglyceride lipase to complete the hydrolysis of TAG (Holm 2003). ATGL and HSL work in series, with ATGL having a higher affinity for TAG and HSL a higher affinity for the next step in TAG hydrolysis, diacylglycerol. Thus, ATGL is the 'initiator' lipase (Miyoshi et al. 2007) for NE-stimulated lipolysis and may be rate limiting (Haemmerle, Lass, Zimmermann, Gorkiewicz, Meyer, Rozman, Heldmaier, Maier, Theussl, Eder, Kratky, Wagner, Klingenspor, Hoefler, & Zechner 2006). ATGL activity, unlike that of HSL and perilipin A, is not dependent on PKA phosphorylation, but is dependent on the cofactor, CGI-58 (Kershaw et al. 2006; Lass et al. 2006). ATGL activation, however, also is dependent on PKA-induced perilipin A phosphorylation (Miyoshi, Perfield, Souza, Shen, Zhang, Stancheva, Kraemer, Obin, & Greenberg 2007).

Sympathetically Driven Phosphorylation of the Adipocyte-Associated Proteins Hormone Sensitive Lipase and Perilipin A Is Important For White Adipocyte Lipolysis and Brown Adipocyte Thermogenesis

By knowing the SNS drive (NETO) to WAT depots, one can only infer that the WAT pads demonstrating increases in NETO contribute to increases in the circulating lipolytic products glycerol and FFAs [*e.g.*, (Brito, Brito, Baro, Song, & Bartness 2007; Brito, Brito, & Bartness 2008)]. Other tissues such as muscle and liver or non-assayed WAT pads could, however, contribute to these TAG metabolites (Newsholme and Leech 1983). To determine which WAT depots contribute to catecholamine-stimulated lipolytic activity, an intracellular marker of lipolysis is needed. Because we know that phosphorylation of perilipin A and HSL (pPerilipin and pHSL, respectively) are integral to NE (sympathetic)-stimulated lipolysis (see directly above), as shown *in vitro* [*e.g.*, (Brasaemle et al. 2004; Gross et al. 2006)], then they could serve as such a lipolytic indicator *in vivo*. Indeed, we recently found that central agonism of melanocortin receptors with MTII increased pPerilipin A and pHSL only in depots showing increased NETO (IWAT and IBAT), but not those where NETO is not increased (RWAT and EWAT; Shrestha, Vaughan, Smith, Song, Baro and Bartness, submitted). This effect is likely via agonism of the melanocortin 4-R subtype (MC4-R) because MC4-R mRNA is highly (~60 %) co-localized on PRV-labeled SNS outflow neurons from brain to WAT (Song et al. 2005b). Therefore, increases in pPerilipin A and/or pHSL can be used as indicators of fat pad-specific catecholamine-induced lipolysis *in vivo*.

Not All Lipid Mobilization Is Via Activation of the Sympathetic Innervation of White Adipose Tissue

Even though it appears that the activation of the SNS innervation of WAT is the principal mechanism underlying lipid mobilization, there appears to be some important exceptions. For example, the lipid mobilizing effects of physiological peripheral doses of leptin are blocked by WAT sympathetic denervation in laboratory mice, but not laboratory rats (Rooks, Penn, Kelso, Bowers, Bartness, & Harris 2005), suggesting another, as yet, undetermined lipolytic mechanism. In this and other regards, the roles of all circulating factors should not be ignored. Atrial natriuretic peptide [ANP; a.k.a. atrial natriuretic factor, atrial natriuretic hormone, atriopeptin] powerfully stimulates lipolysis to the same degree as catecholamine-induced lipolysis (Sengenès et al. 2000), although apparently only in human adipocytes, but not those

from rodents (Sengenès, Berlan, de, I, Lafontan, & Galitzky 2000; Sengenès et al. 2002). Using *in situ* microdialysis to obtain glycerol release rates (*i.e.*, lipolysis), ANP increases lipolytic rate in human abdominal subcutaneous WAT independently of catecholamines (Galitzky et al. 2001). Although appropriately rejected as a primary stimulator of lipolysis in food deprivation, SDs and other lipolytic challenges mentioned above, adrenal medullary EPI stimulates exercise-induced lipolysis independently of NE in humans (de Glisezinski et al. 2009). Finally, insulin is a highly potent and effective lipid regulator in both humans and rodents [*e.g.*, (Lambert et al. 1978; Rudman and Shank 1966)], as prandial and post-prandial increases in pancreatic insulin inhibit lipolysis and promote lipid accumulation in adipocytes as well as promoting glucose uptake into liver and muscle for conversion to glycogen (Newsholme & Leech 1983).

White Adipose Tissue Has Sensory Innervation

WAT has significant sensory innervation, as revealed by application of the anterograde tract tracer True Blue into laboratory rat IWAT or DWAT labels pseudounipolar neurons in the dorsal root ganglia (DRG; (Fishman and Dark 1987)). In support of these tract tracing data, immunohistochemical labeling of the sensory nerve-associated neuropeptides substance P (Fredholm 1985) and calcitonin gene-related peptide [CGRP; (Hill et al. 1996)] occur in laboratory rat WAT (Giordano et al. 1996) and CGRP in Siberian hamster IWAT and EWAT (Foster & Bartness 2006; Shi et al. 2005; Shi and Bartness 2005). Co-cultured 3T3-L1 cell line adipocytes with DRG neurons develop *en passant* neural connections, as revealed by electron microscopy (Kosacka et al. 2006) reminiscent of the type of nerve terminals that characterize the sympathetic innervation of WAT adipocytes revealed by electron microscopy noted above (Slavin & Ballard 1978). Collectively, these data strongly support the neuroanatomical reality of the sensory innervation of WAT, but do not identify: 1) the spinal cord and brain sites that receive this sensory input and 2) the type of sensory nerve terminals that occur in the tissue itself. Identification of the former requires transneuronal tract tracing studies, while the understanding latter requires electron microscopy of WAT.

Using viral transneuronal tract tracing methodology, this time with the H129 strain of herpes simplex virus-1 (generously donated by Dr. Richard Dix, Georgia State University), and borrowing from the approach used to define the central sensory circuits from the stomach to the brain (Rinaman and Schwartz 2004), we recently defined the central sensory circuits from WAT to brain (Song, Schwartz, & Bartness 2009a) and preliminarily from BAT as well (Song and Bartness 2007). Whereas PRV travels strictly in a retrograde direction, H129 is an anterograde traveling virus going from the WAT pad site of inoculation, in our case from IWAT, to the soma of the pseudounipolar sensory neurons in the DRG where it replicates, and continues its anterograde journey to and within the brain to label the central sensory circuits from WAT. We found infected cells across the neuroaxis after H129 injection into IWAT in Siberian hamsters (Song & Bartness 2007). Interestingly, the destinations or components of these afferent projection circuits included a remarkable number of origins of the SNS efferent outflow from brain to WAT we identified previously using PRV (Bamshad, Aoki, Adkison, Warren, & Bartness 1998; Song, Jackson, Harris, Richard, & Bartness 2005b; Song & Bartness 2001). Thus, to date, it appears that WAT only has spinal afferents. In support of this suggestion, injection of cholera toxin-B, a retrograde tracer, into RWAT labeled a small number (~4-7) of cells in the nucleus gracilis of the brainstem, indicating input from spinal afferents (Kreier, Kap, Mettenleiter, van, van, V, Kalsbeek, Sauerwein, Fliers, Romijn, & Buijs 2006). Those data, however, are difficult to interpret, as these studies found no labeling in the dorsal horn of the spinal cord, and the DRG was not examined (Kreier, Kap, Mettenleiter, van, van, V, Kalsbeek, Sauerwein, Fliers, Romijn, & Buijs 2006). Traditionally, information relayed through the ascending spinothalamic and spinocerebellar circuits are thought to relay pain and proprioceptive information, respectively (Willis and Coggeshall 1991). This appears to be a

somewhat oversimplified categorization of sensory inputs to the brain. For example, SNS afferent soma reside in the DRG of the thoracic and more rostral lumbar spinal levels (Cervero and Foreman 1990), as we found, they contain non-nociceptive (*i.e.*, non-pain) visceral receptors involved in the reflexive control and/or homeostasis (Cervero & Foreman 1990). In the present context, these visceral afferents may convey sensory information associated with lipolysis, as our 2DG electrophysiological evidence would suggest (see below). Thus, this appears to be a rather novel function of spinal afferents in general, and WAT afferents specifically.

Sensory Innervation of White Adipose Tissue May Monitor and Modulate Lipolysis

What is the function of the sensory innervation of WAT? Although this question lacks a definitive answer, several roles can be suggested. One possibility is that the sensory nerves comprise a neural conduit extending from adipose tissue to the brain, to inform it of lipid reserves. Currently, many believe that this function is served by CNS access to circulating leptin, the largely adipose-derived cytokine (Zhang et al. 1994) whose plasma levels are roughly proportional to adipocyte size [*i.e.* the size of their TAG stores; for review see: (Jequier and Tappy 1999)]. Alternatively, leptin may also act on peripheral sensory neurons that, in turn, project to the brain. The functional long form of the leptin receptor (Ob-Rb) is expressed by nodose ganglion neurons in laboratory rats and humans (Burdyga et al. 2003). Thus, leptin released from adipocytes could bind to Ob-Rb located on visceral afferent cell bodies as well as on the rat vagal nerve trunk (Buyse et al. 2001). Vagal afferent signaling of leptin has been demonstrated in the cat gastrointestinal system where leptin activates vagal afferent intestinal mechanoreceptors (Gaige et al. 2002; Gaige et al. 2004; Peters et al. 2006). Furthermore, local intra-WAT pad microinjection of leptin into laboratory rat EWAT provokes dose-dependent increases in the multiunit firing rate of sensory nerves emanating from the fat pad (Nijima 1998; Nijima 1999) and, moreover, elicits increases in sympathetic nerve activity in the contralateral EWAT pad suggestive of a reflex arc (Nijima 1999). In addition, peripheral leptin increases the sympathetic drive ([NETO] to WAT in laboratory mice (Penn et al. 2006) and sympathetic nerve electrophysiological activity in laboratory rats [(Shiraishi et al. 1999)]; *c.f.*, (Collins et al. 1996)]. Collectively, these data suggest that WAT sensory nerves respond to leptin.

We postulate that leptin afferent nerve signaling reaching the central origins of SNS outflow to WAT via WAT sensory spinal afferents could, in turn, trigger increases in sympathetic drive to WAT and thereby initiate lipolysis in feedback fashion. Increases in β_3 -adrenoceptor stimulation, known to inhibit leptin secretion from adipocytes [*e.g.*, (Giacobino 1996)], could thereby reduce leptin-induced sensory nerve negative feedback to SNS outflow sites resulting in reductions in SNS drive to WAT. Although this may seem somewhat farfetched, we have recent preliminary data that can be interpreted as support for this notion. First, if we inject both PRV and H129 into a WAT pad, we find double-labeled (double-infected) neurons in several brain areas demonstrating the neuroanatomical reality of such SNS-sensory feedback loops (Song, Schwartz, & Bartness 2009a). Second, we find Ob-Rb mRNA co-localized on DRG pseudounipolar neurons innervating WAT [sensory afferents; (Song, Schwartz, & Bartness 2009a)]. Finally, it appears that WAT sensory afferents sense increases in lipolysis. That is, glucoprivation following systemic injection of the non-metabolizable glucose analog, 2-deoxy-d-glucose (2DG), increases the SNS drive to WAT (Brito, Brito, & Bartness 2008) and also increases multiunit electrophysiological activity from decentralized WAT afferent nerve bundles (Song, Schwartz, & Bartness 2009a), an effect blocked by pretreatment with the pan β -adrenoceptor blocker, propranolol (Schwartz and Bartness, unpublished observations).

If WAT sensory nerves respond to some aspect of lipolysis, as suggested above, what is being sensed? Among several possibilities are the products of lipolysis (glycerol, FFAs) or SNS-drive associated factors such as prostaglandin E₂ (PGE₂). In terms of the latter, PGE₂ is released by WAT with SNS nerve stimulation, as well as after incubation of white adipocytes with NE and from isolated adipocytes harvested from food deprived laboratory rats (Shaw and Ramwell 1968). In primary cultures of adult laboratory rat DRGs, PGE₂ stimulates cAMP accumulation and may serve to sensitize sensory neurons (Smith et al. 1998). PGE₂, among other prostanoids, also increase the electrophysiological activity of isolated laboratory rat vagal nerve fibers (Smith, Amagasu, Eglen, Hunter, & Bley 1998). Taken together, these data suggest that PGE₂ or other prostanoids could similarly activate WAT sensory nerve electrophysiological activity when released as a consequence of SNS-induced lipolysis. Gastrointestinal extrinsic afferent nerves in sheep (Cottrell and Iggo 1984a; Cottrell and Iggo 1984b), laboratory rats (Lal et al. 2001) and cats (Melone 1986) are responsive to fatty acids (FAs). In cats, one subpopulation of gut afferents is activated by short chain FAs and glycerol, whereas another is stimulated by long chain FAs (Melone 1986) suggesting that at least two distinct receptors for these lipolytic products exist in the gut. Thus, while the lipolytic stimuli sufficient to activate WAT sensory nerves are presently unknown, some reasonable candidates exist, including PGE₂ (Shaw & Ramwell 1968), the lipolytic products glycerol and FAs, and possibly factors that correlate with the lipid content of the adipocytes, such as leptin discussed above. Regardless of which of these factors associated with sympathetic drive and/or lipolysis might be sensed, we posit that paracrine, rather than endocrine, secretions of white adipocytes triggers local WAT sensory nerve activity that in turn, excites DRG neurons. Furthermore, we suggest this sensory information plays a critical role in determining fat pad-specific differences in depot size, lipid mobilization and other metabolic responses.

Some of the predicted characteristics of WAT sensory neurons might be inferred from similar afferents in other tissues. For example, peripheral tissues, including WAT, contain small diameter, unmyelinated sensory nerves, which typically express transient receptor potential vanilloid type 1 (TRPV1) protein, a non-selective cation channel and is highly expressed in such sensory neurons (Caterina et al. 1997). TRPV1 appears to serve as a molecular integrator of noxious stimuli, such as heat and low pH (Pingle et al. 2007) and is stimulated by endogenous endovanilloids that include three classes of lipids: a) unsaturated N-acyldopamines (Toth et al. 2003), b) lipoxygenase products of arachidonic acid (Cravatt et al. 1996) and c) the endocannabinoid anandamide (Ross et al. 2001). Local application of high concentrations of the pungent ingredient of hot chili peppers, capsaicin [as well as by other plant toxins, the most potent of which is resiniferatoxin (RTX) (Szallasi et al. 1999)] binds to TRPV1 receptors. At high concentrations, capsaicin will permanently ablate these sensory neurons, a technique used frequently to study the role of the unmyelinated vagal sensory neurons of the gut [*e.g.*, (Berthoud and Powley 1993; Raybould et al. 1990)]. Interestingly, TRPV1 deficient mice have reduced adiposity (Motter and Ahern 2008). Although the mechanism underlying the decreased body fat of TRPV1 knockout mice is not known, one of the two postulated functions of WAT sensory nerves is to provide feedback to the central origins of the sympathetic drive to WAT (see above). In the absence of such feedback it might be predicted that once triggered, the sympathetic drive to WAT and thus, ultimately, increased lipolysis would proceed unabated until other possible feedback mechanisms came into play (*e.g.*, end product inhibition, leptin signaling via sensory nerves and/or humorally). Thus, TRPV1 may be an important mediator of the WAT sensory negative feedback control of adiposity. CGRP also is found in a subpopulation of capsaicin-sensitive A and C primary afferents, and is typically released locally in response to noxious stimuli [for review see: (Lundberg et al. 1992)]. Other functions, such as modulating sympathetic drive to adipose tissue proposed here, could be possible as well. For example, CGRP and the structurally-related adrenomedullin are both found in sensory nerves (Miriel et al. 2009), although the latter has not been shown for the sensory innervation of WAT. Both bind to calcitonin receptor-like receptor (CLR) and the receptor activity-

modifying protein-1 (RAMP1). CGRP, adrenomedullin and RAMP1 not only are found on sensory nerves, but have a presence in human abdominal fat, as well as isolated adipocytes and preadipocytes (Gupta et al. 2007). Osaka *et al.* (Osaka et al. 1998) postulated that CGRP inhibits NE-induced increases in BAT thermogenesis. Similarly, it is possible that CGRP, or adrenomedullin, might modulate NE-induced WAT lipolysis. Although Osaka *et al.* (Osaka, Kobayashi, Namba, Ezaki, Inoue, Kimura, & Lee 1998) believed the inhibition of the NE stimulation of thermogenesis occurred by CGRP acting at its receptors on brown adipocytes, it is possible that CGRP and/or adrenomedullin could act on sympathetic nerves innervating WAT to modulate local NE release. Substantiation of this speculation would require not only identification of CLR/RAMP1 on the sympathetic nerves innervating WAT, but also demonstration that local CGRP/adrenomedullin application alters the electrophysiological activity of the WAT sympathetic efferents. Alternatively, CGRP could act directly at the aforementioned CLR receptors on adipocytes to attenuate the lipolytic effects of SNS NE outflow to WAT.

As CGRP is a potent vasodilator, its role in adipose tissue regulation may result from altered hemodynamics as well. Upon WAT sensory nerve stimulation, CGRP release from these WAT afferents would promote local vasodilatation and thereby increase blood flow. Such an increase could lower local concentrations of adipose factors that have been proposed to drive WAT afferents, such as leptin, PGE₂, FFA and glycerol. In addition, such vasodilatation could promote the influx of circulating nutrients and hormones to WAT that would modify oxidative metabolism and adipose tissue gene expression. Both of these speculations represent consequences of local CGRP release from WAT sensory nerves that would contribute to the ability of WAT to signal CNS sites involved in the negative feedback control of lipid homeostasis.

Another Function of the Sensory Innervation of White Adipose Tissue May Inform the Brain of Lipid Energy Stores

In order to test the necessity of WAT sensory nerves in the control of WAT metabolism, they must be selectively destroyed. Therefore, we injected capsaicin locally into WAT to produce selective sensory denervation of capsaicin-sensitive unmyelinated fibers (Foster & Bartness 2006; Shi, Song, Giordano, Cinti, & Bartness 2005; Shi & Bartness 2005). Specifically, locally injected capsaicin produces a marked reduction in CGRP-immunoreactivity (ir), but not TH, a proven marker of sympathetic nerves (Foster & Bartness 2006; Shi, Song, Giordano, Cinti, & Bartness 2005; Shi & Bartness 2005). Unlike SNS denervation of WAT, which results in an impressive increase in WAT mass due to increases in fat cell number [adipocyte proliferation (Bowers, Festuccia, Song, Shi, Migliorini, & Bartness 2004; Cousin et al. 1993; Foster & Bartness 2006; Shi, Song, Giordano, Cinti, & Bartness 2005; Youngstrom & Bartness 1998)], sensory denervation does not affect this response; however, it slightly increases WAT cell size (Foster & Bartness 2006).

We tested WAT sensory nerve function by utilizing the lipectomy model whereby the hypothetical body fat regulatory system is directly challenged by surgical removal of specific WAT depots [for review see: (Mauer et al. 2001)]. Lipectomy triggers a compensatory increase in the mass of the remaining unexcised WAT pads seen across a number of species including laboratory mice and rats, lambs, ground squirrels and Syrian and Siberian hamsters, as well as suggestive evidence in humans [for review see: (Mauer, Harris, & Bartness 2001)]. The trigger for the compensatory increases in the growth of the non-excised WAT pads has always been puzzling. With the discovery of leptin (Zhang, Proenca, Maffei, Barone, Leopold, & Friedman 1994), an obvious possibility was a decrease in circulating leptin concentrations that should accompany the lipectomy-induced decreases in total body fat. Indeed, serum leptin concentrations are decreased in lipectomized Siberian hamsters 1 and 7 week post surgery

(Dailey and Bartness 2008), and in *ob/ob* and *db/db* mice after 1 week post lipectomy (Harris et al. 2002). In humans, there is a mixed result with initial transient increases and then subsequent decreases (Busetto et al. 2008) with a similar finding of an increasing trend in Zucker *fa/fa* (Schreiber et al. 2006); both effects are ascribed to initial inflammatory responses due to the surgery. One week after lipectomy serum leptin concentrations were significantly decreased in another human study, however (Talisman et al. 2001). Although far from perfect, it does appear that circulating leptin concentrations are, more often than not, decreased after lipectomy. An intact leptin signaling system, however, is not necessary for the lipectomy-induced compensation, because lipectomized *ob/ob* or *db/db* compensate for the removed WAT by increasing the mass of other fat pads similarly to their sham lipectomized counterparts (Harris, Hausman, & Bartness 2002). Thus, another signal must be involved in this response.

With lipectomy, the sensory nerves innervating WAT are inevitably destroyed. Potentially, therefore, the absence of a sensory signal from the removed WAT pad could trigger the lipectomy-induced increases in the mass of the non-excised fat pads. Because of the ability to locally and selectively denervate WAT sensory nerves, as discussed above, we injected capsaicin into Siberian hamster EWAT that resulted in a verified selective WAT sensory nerve loss (decreased CGRP-ir but not TH-ir) and compared the response of the non-injected WAT pads to that of real EWAT lipectomized hamsters (Shi & Bartness 2005). Not only did the capsaicin-injected hamsters increase the masses of the non-injected WAT depots similarly to real lipectomized hamsters, despite the absence of a true lipid deficit, but the size of these WAT pads increased above that of the real lipectomized hamsters and did so to a degree that approximated the lipid deficit if the EWAT pads had actually been removed (Shi & Bartness 2005). These data, therefore, strongly suggest that at least one of the functions of WAT sensory nerves is to inform the brain of WAT lipid levels. In support of this, pseudounipolar sensory nerves that innervate IWAT, as determined by H129 labeling following inoculation of that WAT, express OB-Rb mRNA (Song et al. 2009b).

Concluding Remarks

We have reviewed some of the information regarding the SNS and sensory innervation of WAT, as well as suggestions of the interaction of these two innervation types. With regard to the sensory innervation of WAT, more seems unknown than known about this aspect of adipose tissue innervation. We propose that the sensory innervation of WAT could form two possibly related functions. The first, as evidenced by the ability of capsaicin EWAT sensory denervation to mimic lipectomy-like increases in the mass of non-injected fat pads (Shi & Bartness 2005), may be to inform the brain of fat stores. Whether this holds for all fat pads, or just those associated with the gonads (EWAT here, parametrial WAT has not been tested in females), is unknown. That is, although it is most common to consider that total body fat is regulated in some manner, instead it may be the brain senses the amount of lipid stores in fat depots that appear critical for reproduction – gonad-associated fat. For example, removal of the EWAT pad abolishes testicular spermatogenesis in laboratory rats (Srinivasan et al. 1986). In Syrian hamsters, EWAT lipectomy, but not removal of even larger WAT depots (both IWAT pads creating twice the lipid deficit) also abolishes spermatogenesis and it cannot be restored by EWAT removal and replacement remote from the testes (Ye, Huddleston, Clancy, Harris and Bartness, in preparation). The EWAT lipectomy-triggered inhibition of spermatogenesis is not due to damage to the innervation of the testes associated with fat pad removal as testicular denervation does not produce this effect (Ye, Huddleston, Clancy, Harris and Bartness, in preparation). Lipectomy-induced increases in food hoarding by Siberian hamsters (Wood and Bartness 1997) is most marked by EWAT removal, and largely not increased by lipid deficits produced by IWAT removal nor is it increased further by EWAT + IWAT removal (Dailey & Bartness 2008). How the WAT afferents sense the presence of the WAT pads/lipid stores is

unknown, but we hypothesized that it could be via factors secreted by white adipocytes such as FFA, glycerol, leptin or PGE₂.

The second possible function of the sensory innervation of WAT is one of interaction with the SNS innervation or consequences of SNS activation to WAT. As noted above, glucoprivation-induced increased IWAT NETO is accompanied by increased IWAT multiunit sensory nerve electrophysiological activity (Song, Schwartz, & Bartness 2009a). Furthermore, also noted above, preliminary data using the SNS transneuronal tract tracer PRV injected into IWAT along with injection of the sensory nerve transneuronal tract tracer H129 results in dually-infected neurons in several brain sites highly suggestive of complete SNS-sensory neural feedback loops (Song, Smith B, Nguyen, & Bartness 2009b). We also note preliminarily that if the sensory nerve tracer H129 is injected into IWAT and the SNS tracer PRV is injected into IBAT, dually infected neurons also are seen in the brain (Song, Smith B, Nguyen, & Bartness 2009b) highly suggestive of an integration of sensations from WAT that could alter SNS drive to BAT to affect energy balance. Finally, we note that CGRP/ adrenomedullin released from WAT sensory nerves could modulate the SNS drive to WAT directly and/or at adipocyte CRP receptors, thereby affecting lipolysis.

We hope that this review stimulates the reader's thinking to extend beyond the traditionally accepted roles of circulating factors in the control of body fat levels to include the involvement of the SNS and sensory innervations of WAT in the control of lipid stores.

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