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## Leptin stimulates sympathetic axon outgrowth

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### Abstract

The neurohormone leptin regulates energy homeostasis. Circulating levels of leptin secreted by adipose tissue acts on hypothalamic neurons in the brain leading to decreased appetite and increased energy expenditure. Although leptin signaling in the central nervous system (CNS) is fundamental to its ability to regulate the body's metabolic balance, leptin also has a variety of effects in many peripheral tissues including the heart, the liver, and the sympathetic nervous system. Leptin stimulation of the hypothalamus can stimulate glucose uptake via the sympathetic nervous system in heart, muscle, and brown adipose tissue. Leptin receptors (Ob-Rb) are also expressed by peripheral sympathetic neurons, but their functional role is not clear. In this study, we found that leptin stimulates axonal growth of both adult and neonatal sympathetic neurons *in vitro*. Leptin stimulates acute activation of the transcription factor STAT3 via phosphorylation of tyrosine 705. STAT3 phosphorylation is required for leptin-stimulated sympathetic axon outgrowth. Thus, circulating levels of leptin may enhance sympathetic nerve innervation of peripheral tissues.

### Keywords

Leptin; axon outgrowth; obesity; cardiac arrhythmia; STAT3

### Introduction

The neurohormone leptin controls energy homeostasis by relaying signals from adipose cells to the brain. Genetic studies have shown that removing leptin, or its receptor, from the central nervous system results in animals that are morbidly obese [1–3]. Not surprisingly, the majority of work in this field has been focused on understanding leptin's central effects

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[4–7]. However, leptin is secreted into the circulation and leptin receptors (Ob-Rb) are found in many peripheral targets including heart, liver, muscle, lungs, adrenals, lymph nodes, and the sympathetic nervous system [8–12]. It is well known that central neurons stimulated by leptin activate the sympathetic nervous system [13–18]. In addition, postganglionic sympathetic neurons express Ob-Rb and therefore can respond directly to changing leptin levels [19, 20]. However, the effect of direct stimulation of sympathetic neurons by leptin has not been characterized.

Many studies show that obesity exacerbates cardiovascular disease states. Recently, we found that obese rats had increased axon growth and developed sympathetic hyperinnervation of the heart compared to rats fed normal chow [21]. A potential mediator could be leptin, which is elevated during the obese state. Interestingly, Ob-Rb is a member of the cytokine receptor class I super family and is most closely related to gp130 receptor subunit [22]. We recently reported that gp130 cytokines are required for maximal axon outgrowth in sympathetic neurons [23]. Due to leptin's closely related homology to gp130 cytokines, it is possible they share similar mechanisms as well. Leptin binding to Ob-Rb activates Jak2—STAT3 and MAPK signaling pathways [24] [25]. However, STAT3 activation is required for leptin's central effects regulating body weight and energy homeostasis [26, 27] [28]. Indeed, the chronic inflammatory state associated with obesity and cancer is characterized by increased circulating cytokines and activated STAT3 levels [29]. Phosphorylation of STAT3 is required for sympathetic axon outgrowth [23], but leptin activation of STAT3 has not been tested in this paradigm.

In the present study, we tested the hypothesis that leptin stimulation of sympathetic neurons regulates axon growth. We found that leptin increased sympathetic axon growth in adult and neonatal explants. This effect required phosphorylation of STAT3 on tyrosine 705. Thus, similar to related gp130 cytokines, leptin stimulated sympathetic axon outgrowth requires activation of STAT3. Leptin-stimulated increased sympathetic tone to peripheral tissue targets may help explain how circulating leptin levels contribute to obesity related diseases in humans.

## Materials and Methods

### Materials

Matrigel™ was purchased from BD Biosciences (San Jose, CA). CNTF was from Preprotech (Rocky Hills, NJ). Recombinant mouse leptin was purchased from R & D Systems (Minneapolis, MN). Nerve growth factor (NGF) was purchased from Austral Biologicals (San Ramon, CA). Dispase was purchased from Boehringer Mannheim (Indianapolis, IN). Collagenase type II was purchased from Worthington Biochemicals (Freehold NJ). Nitrocellulose membranes were from Schleicher & Schuell (Dassel, Germany). Protease inhibitor cocktail, phosphatase inhibitor cocktail (#2 & #3), and poly-L-lysine were purchased from Sigma-Aldrich (St. Louis, MO). Bovine Serum Albumin-Fraction V (BSA) was from Thermo Fischer Scientific (Waltham, MA). STATi/Statitic (STAT3 phosphorylation inhibitor) was from Calbiochem (Darmstadt, Germany).

## Animals

Pregnant adult Sprague–Dawley rats were obtained from Charles River Laboratories (Wilmington, MA). Animals were anesthetized using isoflurane and decapitated prior to harvest of ganglia. All procedures were approved by the OHSU Institutional Animal Care and Use Committee and comply with the Guide for the Care and Use of Laboratory Animals published by the National Academies Press (8th edition).

## Western Blotting and Antibodies

Total STAT3 (#9132) and phospho-STAT3 (Tyr705) (#9131) antibodies were from Cell Signaling Technology (Danvers, MA). Species-specific secondary antibodies conjugated to horseradish peroxidase were from Pierce (Rockford, IL). Neonatal sympathetic neurons (postnatal day 1–4) were dissociated and cultured in serum free medium as described previously [23]. Cells were treated with 200 ng/ml leptin for the times specified, and lysates subjected to western blotting as described previously [23]. Bands were captured using the ChemiDoc™ XRS+ system and image lab software (Bio-Rad Laboratories, Hercules, CA). Immunoreactive bands were quantified using Image Lab Software 4.0.1 from Bio-Rad Laboratories.

## Sympathetic axon outgrowth from SCG explants

Both SCGs were removed from neonatal (postnatal days 1–4) or adult rats, halved, and explanted into a 12-well tissue culture plate and covered with 35  $\mu$ L Matrigel (BD Biosciences, San Jose, CA). Serum-free DMEM/F12 media (Life Technologies, Grand Island, NY) + 1% penicillin/ streptomycin was layered over the solidified Matrigel, and wells were treated with 2ng/mL NGF. Culture plates were placed in a humidified incubator of 95% O<sub>2</sub>/5% CO<sub>2</sub> at 37°C. Axon outgrowth was visualized with phase contrast microscopy, and axon length was measured in the images using Nikon Elements AR 3.0 software (Melville, NY). Initial images for “time-zero” were taken 24-hrs after plating. At time-zero, explants were either treated with vehicle or leptin (Sigma, 100 ng/mL), and additional images were taken 24-hrs later (Time 24-hr). For the Static experiments neonatal ganglia were used and Static (20 $\mu$ m) was added to all explants for 6 hrs after the 24 hr pictures were taken. The rate of axon outgrowth ( $\mu$ m/hr) was calculated from the difference in axon growth length before and after treatment, and then dividing this value over the duration of the treatment (24hr for leptin or 6hr for Static). Multiple sites of growth (3–6) from were taken for each explant and averaged. Each condition had 4 replicates and these experiments were performed 4 times. For adult explants, SCGs were bisected and axon growth was measured in two wells for each animal (N=3). The values from each well were averaged to determine the mean rate of axon outgrowth for each animal.

## Statistics

Student’s T test was used for a comparison between two groups. Statistics were carried out using Prism 5.0 (GraphPad Software, La Jolla, CA).

## Results

### Leptin enhances sympathetic outgrowth

To test the hypothesis that leptin stimulates sympathetic axon outgrowth, we measured axon outgrowth from explanted SCGs from adult rats ( $n=3$ ) treated with either vehicle or leptin. At time-zero, axon length was similar between SCGs treated with vehicle ( $205\pm 30\ \mu\text{m}$ ) or leptin ( $253\pm 12\ \mu\text{m}$ ). After the 24-hr treatment period, axon length was longer in leptin ( $701\pm 90\ \mu\text{m}$ ) compared to vehicle ( $484\pm 30\ \mu\text{m}$ ), and the rate of axon outgrowth was 39% higher in SCG treated with leptin compared to vehicle-treated SCG (Figure 1A–C,  $p<0.05$ ).

### Leptin stimulates phosphorylation of STAT3 in sympathetic neurons

To investigate the signaling pathway activated by leptin in sympathetic neurons, we examined levels of STAT3 phosphorylation using SDS-PAGE. We treated neonatal sympathetic neurons with leptin (200ng/ml) over a 30 minute time course using ciliary neurotrophic factor (CNTF) as a positive control (Figure 2A). Leptin stimulated phosphorylation of STAT3 on Y705 at all time points tested. Leptin stimulated a maximum 163.72% increase in the ratio of phospho-STAT3 to total STAT3 at 30 min and we used this time point to test the STAT3 inhibitor Stattic's ability to block leptin-stimulated STAT3 phosphorylation. We pretreated sympathetic neurons with Stattic and then stimulated the neurons with leptin for 30 minutes. Stattic pretreatment blocks basal and leptin-stimulated STAT3 phosphorylation (Figure 2B).

### STAT3 phosphorylation is required for sympathetic axon outgrowth

To test the role of phosphorylated STAT3 in sympathetic axon outgrowth, we measured axon outgrowth of neonatal SCG explants. We compared untreated ( $n=4$ ,  $37.48\pm 2.30\ \mu\text{m}$ ) to leptin treated ( $n=4$ ,  $48.13\pm 1.42\ \mu\text{m}$ ) explants. After the 24-hr treatment period, leptin stimulation increased growth rates 28.41% compared to untreated explants (Figure 3A,  $p<0.01$ ). After 24 hrs of leptin treatment, Stattic was added to all explants for 6 hrs to test the requirement of STAT3 phosphorylation in leptin-stimulated axon outgrowth. Stattic blocked both untreated ( $-94.61\pm 1.68\%$ ) and leptin-stimulated ( $-101.8\pm 5.92\%$ ) axon outgrowth equally (Figure 3B,  $p=0.2888$ ).

## Discussion

In 2008, over 1.4 billion people suffered from obesity-related health issues worldwide (World Health Organization, web reference, 2013). A key regulator of energy homeostasis is the neurohormone leptin. Plasma levels of leptin signal the state of fat stores to the brain and energy regulation is adjusted accordingly. Sympathetic nerve activation is one of the major tools the brain uses to control metabolic states of peripheral tissues. Thus, leptin effects on the nervous system have been investigated thoroughly in the brain, but fewer studies have examined peripheral effects of leptin. In this study, we show that direct stimulation of post-ganglionic sympathetic neurons with leptin promotes axon outgrowth. In addition, we provide evidence that STAT3 phosphorylation is required for leptin effects on sympathetic outgrowth. Thus, our work identifies a novel mechanism by which circulating leptin regulates sympathetic input to peripheral targets in an obesity paradigm.

The leptin receptor belongs to the class 1 cytokine receptor family [30, 31]. Although there are multiple forms of the leptin receptor, the long form (ObRb) is the best characterized and is the only form that activates STAT3 [32, 33]. The 3 main signaling pathways triggered by leptin are STAT3, MAPKs, and PI3K pathways. Different arms of leptin signaling regulate specific facets of energy homeostasis. Leptin activation of MAPKs has been implicated in combating stress, proliferation, and protection after ischemia reperfusion [34–37]. STAT3 and PI3K pathways are required for central control of feeding behavior [38, 39]. In central neurons, the Ob-Rb, STAT3 and MAPK pathways are required for leptin stimulated axon outgrowth [40]. Recently, we showed cytokine-activated STAT3 mediates sympathetic axon outgrowth in an injury paradigm [23]. Numerous studies implicate STAT3 activation in axon regeneration and outgrowth [23, 41–43]. Since leptin shares homology with the IL-6 cytokine family, and activates STAT3, it is not surprising that it can enhance sympathetic axon outgrowth.

Although obesity is associated with a number of metabolic syndromes, abnormal weight maintenance is clearly linked to cardiovascular disease states. Abnormal leptin expression can alter  $\beta$ 3-adrenergic receptor expression in cardiac myocytes [44] and induce ventricular hypertrophy resulting in higher mortality in mice [45]. Recent studies have shown that animals fed a high fat diet have an increased risk of ventricular arrhythmias and sudden cardiac death [21,46, 47]. Sympathetic hyperinnervation in the heart has been linked to arrhythmias in animals and humans [48, 49]. Interestingly, development of arrhythmias is seen even in the absence of obesity [47], and a high fat diet without overt obesity results in cardiac sympathetic hyperinnervation [21]. A high fat diet increases serum levels of many growth factors including EGF, IGF-1, Insulin, MCP-1, IFN- $\gamma$ , and leptin [50]. In addition, adipose tissue is capable of secreting the classic sympathetic growth factor, NGF [51]. Thus, increased fat intake changes serum levels of multiple nerve growth promoting compounds, but leptin-stimulated sympathetic nerve sprouting may provide an additional mechanism for hyperinnervation of peripheral tissues.

## Conclusions

Leptin signaling in CNS is central to its ability to regulate the body's energy stores, but leptin has a variety of effects in many peripheral tissues including the heart and the sympathetic nervous system. In this study, we demonstrate that leptin stimulates axon outgrowth in adult and neonatal sympathetic neurons. Phosphorylation of STAT3 on Y705 is required for leptin-stimulated axon growth. Circulating levels of leptin may explain sympathetic hyperinnervation of the heart and increased risk of arrhythmias. Given the predominance of obesity today, these findings clearly warrant further study.

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## Abbreviations

<b>STAT3</b>	Signal transducer and activator of transcription 3
<b>Ob-Rb</b>	Long form Leptin Receptor
<b>MAPK</b>	Mitogen-activated protein kinase
<b>SCG</b>	superior cervical ganglion
<b>JAK2</b>	Janus kinase-2
<b>PI3K</b>	Phosphoinositide 3-kinase

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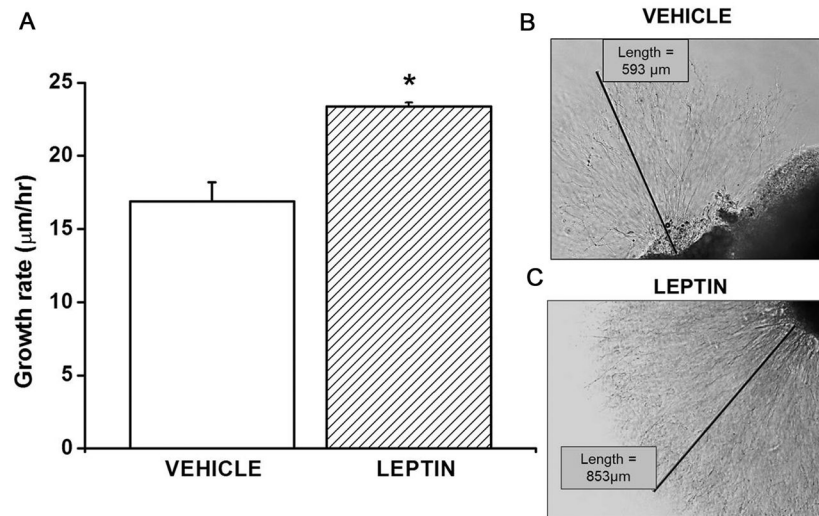
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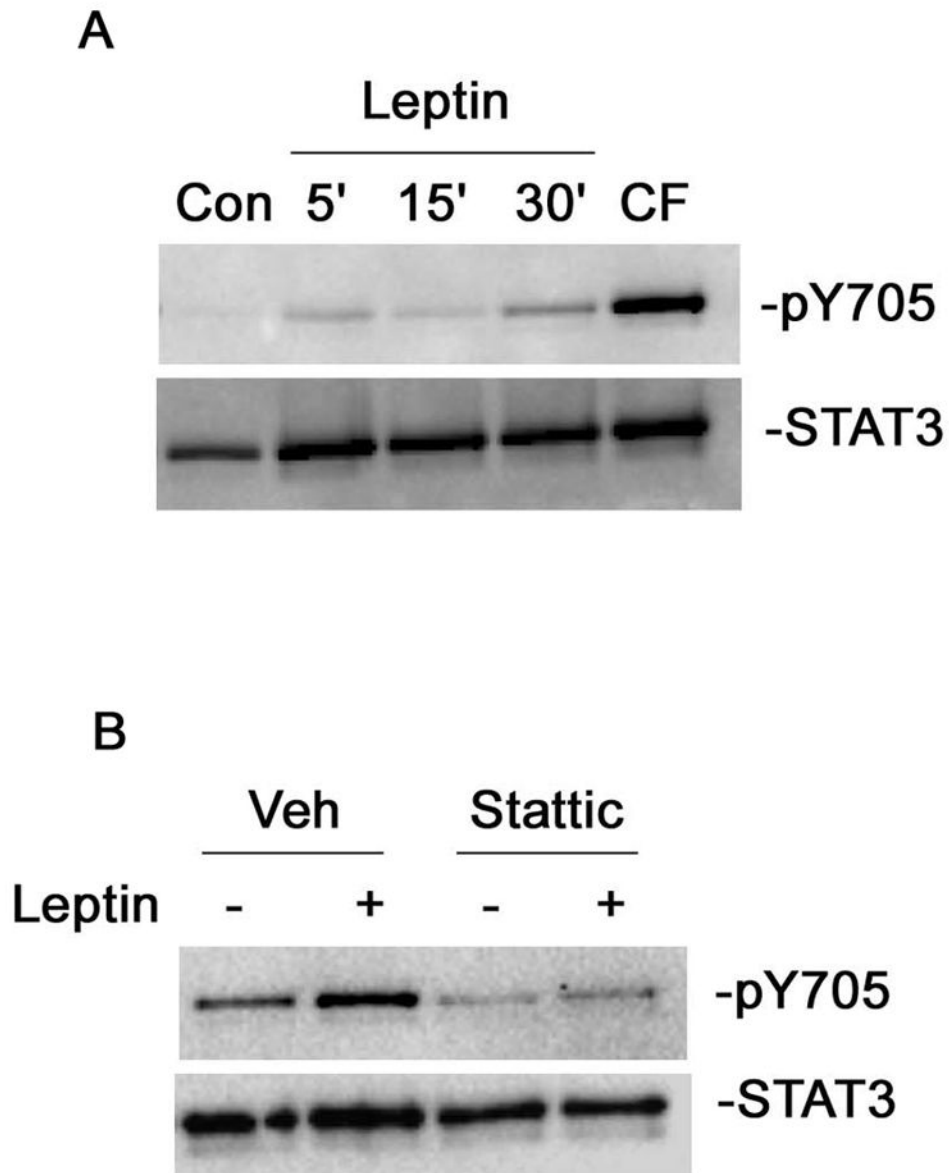


### Highlights

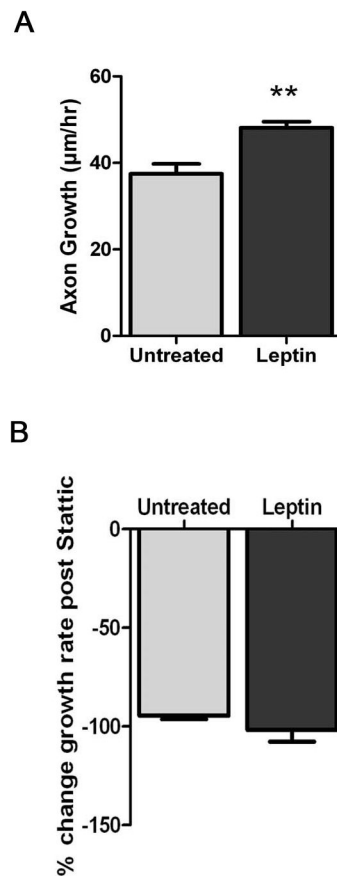
1. Leptin stimulates increased axon growth in sympathetic explants
2. STAT3 phosphorylation is required for leptin-stimulated axon outgrowth
3. This work links a high fat diet to increased risk of cardiac arrhythmias



**Figure 1.** Assessment of axon outgrowth in SCG explants treated with leptin. (A) In adult rats ( $n=3$ ), leptin (100 ng/ml; striped bar) accelerated axon outgrowth in SCG explants compared to vehicle (white bar). (B–C) Photos at Time 24-hr in SCG explants treated with vehicle (B) or leptin (C). Data are mean $\pm$ SEM, \* $p<0.05$ . Three animals were assayed in duplicate ( $N=3$ ).



**Figure 2.** Stattic blocks leptin-stimulated STAT3 phosphorylation. (A) Dissociated sympathetic neurons were stimulated with leptin (200ng/ml) for the indicated time points or CNTF (100ng/ml) for 5min. Phospho-STAT3 (pY705) and total STAT3 levels were measured via western blotting. (B) Dissociated sympathetic neurons were pre-treated with vehicle or Stattic (20 $\mu$ m) for 10min prior to stimulation with leptin (200ng/ml) for 30min. Phospho-STAT3 (pY705) and total STAT3 levels were measured as in A. This is a representative experiment that was repeated at least 3 times.



**Figure 3.**

Leptin stimulates sympathetic axon growth via STAT3. (A) Paired SCG explants were treated with leptin for 24 hours and axon growth rates were calculated. Leptin treated explants ( $n=4$ ) had faster growth rates than untreated controls ( $n=4$ ). Data are mean $\pm$ SEM, \*\* $p<0.01$ . (B) The same explants shown in A were treated with Stattic to block STAT3 phosphorylation. The percent change in growth was similar in both untreated and leptin treated explants after 6 hours. This is a representative experiment that was repeated 4 times ( $N=4$ ).