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Segregation of acute leptin and insulin effects in distinct populations of arcuate POMC neurons

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Abstract

Acute leptin administration results in a depolarization and concomitant increase in the firing rate of a subpopulation of arcuate POMC cells. This rapid activation of POMC cells has been implicated as a cellular correlate of leptin effects on energy balance. In contrast to leptin, insulin inhibits the activity of some POMC neurons. Several studies have described a "cross-talk" between leptin and insulin within the mediobasal hypothalamus via the intracellular enzyme, phosphoinositol-3-kinase (PI3K). Interestingly, both insulin and leptin regulate POMC cellular activity by activation of PI3K, however it is unclear if leptin and insulin effects are observed in similar or distinct populations of POMC cells. We therefore used dual label immunohistochemistry/in situ hybridization and whole-cell patch-clamp electrophysiology to map insulin and leptin responsive arcuate POMC neurons. Leptin-induced Fos activity within arcuate POMC neurons was localized separate from POMC neurons which express insulin receptor. Moreover, acute responses to leptin and insulin were largely segregated in distinct sub-populations of POMC cells. Collectively, these data suggest that cross-talk between leptin and insulin occurs within a network of cells rather than within individual POMC neurons.

Keywords

leptin; POMC; insulin; patch clamp; PI3K; arcuate

Introduction

The adipose-secreted hormone leptin circulates in the plasma at levels which are proportionate to adipose tissue mass (Maffei et al., 1995; Zhang et al., 1994). Since the proposal of the lipostatic hypothesis by Kennedy to the targeted molecular disruption of leptin receptors in the brain a prevalent model of energy homeostasis has developed (Balthasar et al., 2004; Cohen et al., 2001; Kennedy, 1953). The model suggests leptin is not only an adipostatic secreted factor, but rather a metabolic cue which communicates directly to the brain the status of available energy stores (Ahima et al., 1996; Halaas et al., 1995; Schwartz and Porte, 2005; Spiegelman and Flier, 2001; Vaisse et al., 1996). Similarly, the beta-cell derived hormone insulin circulates in proportion to blood glucose levels and mediates some of its effects in the central nervous system (Obici et al., 2002; Woods et al., 1996; Woods et al., 1990; Woods et

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al., 1979). Despite intense investigation, the cellular and molecular mechanisms regarding how leptin and insulin exert their effects remain to be completely delineated.

Considerable attention has centered on the mediobasal hypothalamus as a key site for the integration of leptin and insulin signaling. The results of these studies suggest that both leptin and insulin effects on energy homeostasis necessitate the enzyme phosphoinositol-3-kinase (PI3K) (Carvalheira et al., 2005; Fukuda et al., 2008; Mirshamsi et al., 2004; Morrison et al., 2005; Morton et al., 2005; Niswender et al., 2001; Rahmouni et al., 2003; Zhao et al., 2002). Moreover, acute heterogeneous responses of leptin and insulin require PI3K in proopiomelanocortin (POMC) neurons of the mediobasal hypothalamic arcuate nucleus (Choudhury et al., 2005; Hill et al., 2008; Plum et al., 2006). It has largely been assumed that these data support both direct and opposing actions of leptin and insulin in arcuate POMC neurons. However it is unclear how two different hormones share the same signaling cascade but result in opposite effects on cellular activity. Thus, this study was designed to determine how leptin and insulin may acutely interact within POMC cells of the mediobasal hypothalamus.

Materials and Methods

Subjects

The mice in this study were housed in the University of Texas Southwestern Medical Center Facility. All experiments were carried out in accordance with the guidelines established by the National Institute of Health Guide for the Care and Use of Laboratory Animals, as well as with those established by the University of Texas Institutional Animal Care and Use Committee.

Leptin Administration

Male (4-16 weeks) pathogen free POMC-GFP (Parton et al., 2007; Ramadori et al., 2008) mice expressing humanized *Renilla* green fluorescent protein (hrGFP) under the transcriptional control of the *Pomc* gene and C57Bl6 mice were housed in a light-dark (12 hr on/off; lights on at 7:00 a.m.) and temperature-controlled environment with food and water available ad libitum. C57Bl6 mice were injected with recombinant murine leptin intraperitoneal (n=5, 5.0 mg/kg; provided by A.F. Parlow, Harbor-UCLA Medical Center, Torrance, California, USA; through the National Hormone and Peptide Program) or pyrogen free saline (PFS, n=3) (Sigma). All injections were given between 11 a.m. and 12 p.m.

Electrophysiolgy

Whole cell patch-clamp recordings from POMC neurons maintained in hypothalamic slice preparations and data analysis were performed as previously described (Hill et al., 2008). Briefly, 4- to 16-week old mice were deeply anesthetized with isoflurane and transcardially perfused with a modified ice-cold ACSF (described below), in which an equiosmolar amount of sucrose was substituted for NaCl. The mice were then decapitated, and the entire brain was removed as previously described. After removal, brains were immediately submerged in ice-cold, carbogen-saturated (95% O2 and 5% CO2) ACSF (126 mM NaCl, 2.5 mM KCl, 1.2 mM MgCl2, 2.4 mM CaCl2, 1.2 mM NaH2PO4, 21.4 mM NaHCO3, and 5 mM glucose). A brain block containing the hypothalamus was made. Coronal sections (200–250 μM) were cut with a Leica VT1000S Vibratome and then incubated in oxygenated ACSF at room temperature for at least 1 h before recording. Slices were transferred to the recording chamber and allowed to equilibrate for 10–20 min prior to recording. The slices were bathed in oxygenated ACSF (32° C–34°C) at a flow rate of approximately 2 ml/min.

The pipette solution for whole-cell recording was modified to include an intracellular dye (Alexa Fluor 594) for whole-cell recording: 120-130 mM κ-gluconate, 10 mM KCl, 10 mM

HEPES, 1 mM EGTA, 0.3 mM $CaCl_2$, 1 mM $MgCl_2$, 2–5 mM (Mg)-ATP, and 0.3 mM (Na)-GTP, .03 mM Alexa Fluor 594 hydrazide dye, pH 7.3. Epifluorescence was briefly used to target fluorescent cells, at which time the light source was switched to infrared differential interference contrast imaging to obtain the whole-cell recording (Zeiss Axioskop FS2 Plus equipped with a fixed stage and a QuantEM:512SC electron-multiplying charge coupled device camera). Electrophysiological signals were recorded using an Axopatch 700B amplifier (Axon Instruments), low-pass filtered at 2–5 kHz, digitized at 88 kHz (Neuro-corder; Cygnus Technology), stored on videotape, and analyzed offline on a PC with pCLAMP programs (Axon Instruments). Recording electrodes had resistances of 2.5–5 MΩ when filled with the K-gluconate internal solution. Input resistance was assessed by measuring voltage deflection at the end of the response to a hyperpolarizing rectangular current pulse (400–500 ms of −20 pA). Membrane potential values were compensated to account for junction potential (−8 mV).

Insulin (50 nM, Humulin-R 100 U/ml; Lilly) and leptin (100 nM; provided by A.F. Parlow, Harbor-UCLA Medical Center, Torrance, California, USA; through the National Hormone and Peptide Program) were added to the ACSF for specific experiments. Solutions containing insulin or leptin were typically perfused for 2–4 min. A peptide-induced effect was required to be associated temporally with peptide application, and the response had to be stable within a few minutes. A neuron was considered depolarized or hyperpolarized if a change in membrane potential was at least 2 mV in amplitude. After recording, slices were fixed with 4% formalin in PBS at 4°C overnight. After washing in PBS, slices were mounted onto slides, covered in Vectashield (Vector Laboratories) and cover-slipped to reduce photo-oxidation during visualization with fluorescent light. Cells were then visualized with ApoTome imaging system (Imager Z1; Zeiss) to identify post-hoc the anatomical location of the recorded neuron.

Insulin Receptor Probe production

Probes for insulin receptor (insulinR) were derived from PCR fragments amplified with iTaq DNA polymerase (Bio-Rad, Hercules, CA) from cDNA generated with SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) from total mouse hypothalamic RNA (BD Biosciences, Palo Alto, CA). The PCR products were cloned with the TOPO TA Cloning® Kit for Sequencing (Invitrogen). The insulinR probe spans nucleotides 2448-28944 of Genbank accession number NM_010568. Antisense and sense ^{35}S labeled probes were generated with MAXIscript® In Vitro Transcription Kits (Ambion, Austin, TX) with ^{35}S-UTP. The nucleotide mixture was then digested with DNAase, and the labeled probes were purified and collected by using resin spin columns (GE Healthcare, Piscataway, NJ). The ^{35}S-labeled probes were diluted (10^6 dpm/mL) in a hybridization solution containing 50% formamide, 10 mM Tris-HCl, pH 7.5 (Invitrogen), 1% sheared salmon sperm DNA (Sigma-Aldrich, St. Louis, MO), 5mg tRNA (Invitrogen), 2.5% total yeast RNA (Sigma), 100 mM dithiothreitol, 10% dextran sulfate, 0.6 M NaCl, 0.5 mM EDTA (pH 8.0), 0.1% SDS, 0.1% sodium thiosulphate, and 1× Denhardt's solution (Sigma). Hybridization solution and a coverslip were applied to each slide, and sections were incubated overnight, at 57°C. On the following day, sections were incubated in 0.002% RNase A (Roche Applied Bioscience, Indianapolis, IN) solution and submitted to stringency washes. Sections were then dehydrated and enclosed in X-ray film cassettes with Kodak BioMax MR-2 film (Carestream Molecular Imaging, New Haven, CT) for 3 days. Subsequently, the slides were dipped in Kodak NTB autoradiographic emulsion (Carestream Molecular Imaging), dried and stored at 4°C for 15 days. Slides were developed with Dektol developer and Fixer (Kodak/Carestream Molecular Imaging), counterstained with thionin, dehydrated, cleared in xylenes and coverslipped with Permaslip (Newcomer Supply, Middleton, WI). Control procedures included hybridization with sense probes and tissue pre-treatment with RNAase A (200 μg/ml). No specific hybridization was observed following either control procedure.

Dual label immunohistochemistry/in situ hybridization

The procedure was a modification of that described previously (Liu et al., 2003). Tissue was rinsed with DEPC-treated PBS, pH 7.0 for one hour and in 0.1% sodium borohydride (Sigma) in DEPC-PBS for 15 minutes. Sections were then incubated for 10 minutes in 0.25% acetic anhydride in 0.1M TEA. POMC ^{35}S-labeled riboprobe was generated from cDNA templates as described previously (Cheung et al., 1997; Elias et al., 1999). cRNA probes (POMC and insulinR) were diluted to 10^6 cpm/ml in hybridization buffer as described and applied to series of hypothalamic sections. Sections were hybridized overnight at 50°C. The following day, tissue was rinsed four times in 4× sodium chloride/sodium citrate (SSC) and was incubated in 0.002% RNase A (Roche Applied Bioscience) diluted in 0.5M NaCl, 10mM Tris-HCl, pH 8.0, and 1mM EDTA for 30 minutes at 37°C. Sections were submitted to stringency wash in 0.1× SSC for 60 minutes at 55°C. Series of sections of mice treated with leptin or saline were incubated in anti-Fos antisera (1:20,000, Calbiochem) and processed for standard immunoperoxidase reaction using ABC kit (Vector). Series of sections from male wild-type C57Bl/6 mice were incubated in anti-beta-endorphin antisera (1:10,000, Phoenix Pharmaceutical) and processed for standard immunoperoxidase reaction using ABC kit (Vector). Tissue was mounted onto SuperFrost Plus slides (Fisher Sci) and dehydrated in increasing concentrations of ethanol. After air drying, slides were placed in X-ray film cassettes with BMR-2 film (Kodak) for 1-3 days. Slides were then dipped in NTB2 photographic emulsion (Kodak), dried, and stored in dessicant-containing, foil-wrapped slide boxes at 4°C for 1-3 weeks. Slides were developed with D-19 developer, dehydrated in increasing concentration of ethanol, cleared in xylenes, and coverslipped with Permaslip. Sections were analyzed with a Zeiss Axioplan microscope.

Estimates of Cell Counts

In all procedures, the estimates of cell counts were done as previously described (Elias et al., 1999). Briefly, the relative number of neurons containing leptin-induced Fos-IR and POMC mRNAs was counted in the RCA and Arc using a 10× and 20× objectives. Similarly, the relative number of cells containing B-endorphin that also contained insulinR mRNA in the RCA and Arc was also counted. Silver grains above background in that diameter for POMC or insulin mRNAs were considered double-labeled cells (Fos + POMC or B-endorphin + insulinR). The data were not corrected for double counting because the objects we were counting did not change in size, and section thickness did not vary between groups, any systematic error should be identical for all groups. Hence, as all double-label studies are inherently qualitative, our results are meant to provide relative data, but are not meant to be accurate estimates of absolute cell counts. Differences between leptin- and PFS-treated groups were analyzed by student's t-test.

Production of Photomicrographs

Photomicrographs were produced by capturing images with a digital camera (Axiocam, Zeiss) mounted directly on the microscope (Zeiss Axioplan). Image editing software (Adobe Photoshop) was used to combine photomicrographs into plates. Only the sharpness, contrast, and brightness were adjusted. Cytoarchitectonic details were added by using a camera lucida.

Results

Leptin-induced Fos-IR and insulin receptor mRNA in POMC neurons

We first identified leptin and insulin responsive POMC cells within the mouse mediobasal hypothalamus. The protein Fos has been used as a general marker for cellular activation (Hoffman and Lyo, 2002; Hoffman et al., 1993). Moreover, Fos is a useful marker for measuring leptin activation in POMC neurons (Elias et al., 1999). In order to assess the identity

of neurons expressing Fos after leptin administration, we examined leptin- (n=5) and PFS- (n=3) treated mice perfused 2 hr after treatment. Hypothalamic sections were processed for in situ hybridization histochemistry (ISHH) for POMC mRNAs followed by Fos immunohistochemistry. Similar to what has been described in rats, we found that 34% ($t(df)= 6$, $p < 0.05$ compared to PFS-treated mice, student's t-test) of POMC cells in the retrochiasmatic area (RCA) and up to 25% ($t(df) = 6$, $p < 0.05$ compared to PFS-treated mice, student's t-test) in the arcuate nucleus (Arc) express leptin-induced Fos-IR (Fig 1A). Control (PFS) mice exhibited very little Fos-IR in POMC neurons in the RCA/Arc (4.1 ± 1.6%, n=3; Table 1). Additionally, we also noted a rostrocaudal as well as a mediolateral topographic distribution of POMC cells which express leptin-induced Fos-IR (Fig 1B-E). Specifically, rostral sections exhibited a higher percentage of leptin activated cells in the lateral regions toward the optic tracts, while more caudal levels showed leptin-responsive cells bordering the third ventricle. We next investigated whether POMC cells also expressed insulin receptor. Hypothalamic sections were processed for ISHH for insulin receptor followed by B-endorphin immunohistochemistry (Fig 1F). We found that approximately 28% POMC cells in the RCA and up to 36% in the Arc express insulin receptor (Table 1). Similar to the leptin responsive neurons we observed an anatomic pattern of insulin receptor expression by POMC cells. However, this was a rostrocaudal and mediolateral pattern in which insulin receptor colocalization was adjacent to the third ventricle in rostral sections and less colocalized around the ventricle at more caudal levels (Fig 1F-I). These histological data suggested that leptin and insulin receptors maybe in distinct subpopulations of POMC cells.

Acute effects of leptin and insulin in POMC neurons

To further determine leptin and insulin responsive POMC cells, whole-cell patch-clamp recordings were performed on 117 POMC-GFP neurons anatomically confined to either the RCA or the arcuate nucleus from 41 mice (Fig 2A & B). When K^+ was used as the major cation in the recording pipette, POMC neurons in (n = 117) had a resting membrane potential of −53.4 ± 0.6 mV. Neurons were dialyzed with Alexa Fluor 594 hydrazide dye throughout the recording (Fig 2C). Recorded neurons were identified as GFP-labeled POMC neurons via real-time visualization under fluorescence microscopy and via *post hoc* identification of Alexa Fluor dye (Fig 2D & E). In current clamp mode, insulin (50nM) resulted in a hyperpolarization from rest in 10 of 40 POMC-GFP neurons (25%, -6.3 ± 0.6 mV; n = 10; Fig. 2F). The remaining cells were unaffected in response to insulin (75%, -0.6 ± 0.7mV; table 2). In agreement with previous reports in the mediobasal hypothalamus, the insulin-induced inhibition of POMC neurons was completely reversible in most of the cells recorded (n=9), while only one cell did not recover before the recording was lost (Hill et al., 2008; Spanswick et al., 2000). Moreover, the insulin-induced hyperpolarization was repeatable in subsequent applications in 5 neurons tested (Fig 2G). Insulin-inhibited POMC cells were largely confined to the rostromedial aspect of the RCA and Arc (Fig 3). We did not observe any cells which were inhibited by insulin in the most caudal level of the Arc which is in agreement with our histology suggesting a sparse distribution of insulin receptor in POMC cells at this caudal level. Leptin (100nM) caused a depolarization from rest in 16 of 46 POMC-GFP neurons (35%, +7.6 ± 0.9 mV; n = 16; Fig. 2G). Although one cell was hyperpolarized in response to leptin, the remaining 29 neurons remained unchanged (63%, 0.9 ± 0.8mV; table 2). Similar to reports describing acute leptin activation in regions of the hypothalamus (Claret et al., 2007; Cowley et al., 2001; Dhillon et al., 2006; Hill et al., 2008), the depolarization was long lasting in most of the cells such that the depolarization did not reverse during the recording (n=14). However 2 cells completely recovered membrane potential within 20-30 minutes after washout. Post-hoc identification of leptin-excited POMC cells mirrored histological data obtained in that there appeared to be a rostrocaudal and mediolateral distribution to the responses (Fig 3 and table 2).

Acute leptin and insulin responses in distinct populations of POMC cells

In order to further delineate if POMC cells could respond to both insulin and leptin, identified POMC cells were next assessed for effects on membrane potential following successive application of both peptides. Similar to previous reports which investigated the acute effects of insulin or peptides which activate a Katp conductance in the hypothalamus or brainstem, we found the acute insulin-induced inhibition of POMC cells readily reversible (Hill et al., 2008; Spanswick et al., 2000; Williams and Smith, 2006; Williams et al., 2007) and repeatable in subsequent applications (Fig 2G). Therefore, the membrane potential of identified POMC cells was first monitored in the presence of insulin. Following washout of insulin, changes in cellular activity were then examined in the presence of leptin. Similar to our previous experiments and prior reports, insulin (50nM) hyperpolarized 10 of 31 POMC neurons (32%; -6.0 ± 0.3mV; n=10). After washout of the insulin-induced hyperpolarization, subsequent application of leptin (100nM) failed to result in a change of membrane potential in all neurons which previously hyperpolarized in response to insulin (0.2 ± 0.9mV, n=10). The remaining 21 cells were unresponsive to a single application of insulin (-0.8 ± 0.4mV, n=21). However, subsequent application of leptin (100nM) resulted in a depolarization in 7 of these 21 POMC cells (6.8 ± 1.9mV, n=7). The remaining 14 cells were unresponsive to leptin and insulin. Plotting the location of POMC neurons which responded to either insulin or leptin results in a rostrocaudal division of acute leptin and insulin responses which mirrors that describing leptin-induced Fos-IR and insulinR in POMC cells (Fig 4D).

Discussion

There has been an increasing interest in understanding the actions of insulin and leptin in the central nervous system. Intense investigation showed that insulin and leptin converge in many of the same brain areas to reduce food intake and modify glucose and energy homeostasis (Carvalheira et al., 2005; Morton et al., 2005; Xu et al., 2005). Moreover, both peptides have been shown to have opposite actions on neurons within the mediobasal hypothalamus (Claret et al., 2007; Hill et al., 2008). Interestingly, the acute effects of insulin and leptin require the enzyme PI3-kinase in the arcuate nucleus (Hill et al., 2008; Mirshamsi et al., 2004). Naturally this raises the question of how can two peptides effect cells disparately via the same intracellular mechanism.

Divergent leptin and insulin signaling at the level of the POMC cell

Several reports indicate that as few as 30% or as many as 90% of POMC cells in the arcuate nucleus were excited by leptin (Choudhury et al., 2005; Claret et al., 2007; Cowley et al., 2001; Hill et al., 2008). Similarly, previous studies have reported 50% to 80% of POMC cells are hyperpolarized in response to insulin (Choudhury et al., 2005; Claret et al., 2007; Hill et al., 2008). However reports have not directly assessed the differences between leptin-activated and insulin-inhibited POMC cells. Here we report that leptin resulted in a depolarization in 35% of all POMC neurons from the retrochiasmatic area and arcuate nucleus of the mediobasal hypothalamus. The leptin-induced excitation was observed throughout the rostrocaudal levels of the RCA and Arc (from -0.94mm to -2.30mm bregma). However, there was a higher percentage of leptin-excited POMC cells in the lateral division of the RCA and medial group of POMC cells in the Arc, such that 40-70% of POMC cells were excited by leptin in these topographic regions. Moreover, plotting the leptin-induced activation as measured by Fos-IR resulted in a neuroanatomical pattern analogous to the leptin effects observed on acute cellular excitation. On the other hand, insulin-inhibited POMC cells (25%) were largely localized to the medial divisions of the RCA and rostromedial areas of the arcuate nucleus which is in agreement with the observed distribution of insulin receptor. Together, these data suggest that the variability of leptin-activated and insulin-inhibited POMC neurons previously reported may have been dependent upon the rostrocaudal and/or mediolateral level at which cells were

identified and/or targeted. In support of this, we found it noteworthy that studies which report a large percentage of insulin-inhibited arcuate POMC cells also suggest a lower percentage of leptin-excited cells and vice versa (Claret et al., 2007; Hill et al., 2008; Plum et al., 2006). Thus, the distribution of insulin receptor and acute effects of insulin in POMC cells directly contrast with the description of acute leptin-induced activation of POMC cells and suggests a segregation of insulin and leptin responses in arcuate POMC cells. One technical consideration is that Fos-IR has been considered a measure of cellular activation largely out of association (Elias et al., 2000; Hoffman et al., 1993; Luckman et al., 1994; Sagar et al., 1988). Here we show that ~30% of POMC cells in the mouse express Fos protein in response to leptin. Similarly, ~30% of POMC cells are depolarized in response to leptin. Moreover the topographic distribution of Fos-IR and cellular activation in response to leptin mirror one another. Granted this is not a direct assessment of Fos-IR expression in activated cells, but these data extend previous observations that at least in the arcuate POMC cells Fos-IR directly correlates with leptin-induced cellular activation.

It is important to note that both insulin and leptin are capable of activating intracellular signaling cascades which regulate gene transcription independent of changes in cellular activity (Bates et al., 2003; Fukuda et al., 2008; Hill et al., 2008; Xu et al., 2007). Thus, failure of a POMC neuron to acutely modify cellular activity in response to leptin or insulin does not exclude the possibility that either peptide may bind to its cognate receptor and activate signaling cascades within the POMC neuron examined. Moreover, a recent report suggested that insulin could reverse a leptin-induced depolarization of POMC neurons (Al-Qassab et al., 2009). However this activity was found in only a small number of neurons, supportive of a segregation of leptin and insulin effects. Taken with our data, we suggest that many of the POMC neurons that are acutely activated by leptin are independent of acute inhibition by insulin and vice versa.

Model of leptin and insulin action in arcuate POMC cells

The arcuate POMC cell population has largely been considered a homogeneous cell group. However, we and others have previously shown that POMC cells have varying projection patterns (Baker and Herkenham, 1995; Elias et al., 1999; Elias et al., 1998; Swanson and Kuypers, 1980). In rats, neurons of the RCA which express POMC/CART primarily project caudally to autonomic areas including the dorsal vagal complex and the intermediolateral cell column (IML) (Elias et al., 1998; Swanson and Kuypers, 1980; Zheng et al., 2005). On the other hand, more caudal POMC cells project largely to hypothalamic centers like the paraventricular nucleus – PVN - and the lateral hypothalamic area - LHA (Baker and Herkenham, 1995; Elias et al., 1999). Thus the distribution of leptin and insulin responsive POMC cells is a likely influence for the effects of these peptides on autonomic function.

Recently it was shown that selective deletion of insulin receptors in the POMC cells has little effect on energy balance which may be attributed to the differential expression of insulin receptors in POMC cells and the differing projection patterns of these neurons (Konner et al., 2007). The current study supports a role of insulin in modifying autonomic activity rather than food intake via POMC neurons (Hill and Elmquist unpublished observations; Konner et al., 2007). Perhaps the topography of the leptin-activated cells is less surprising, given the involvement of LHA and PVN melocortin-4 receptors in the acute effects of leptin on energy balance. Since the downstream targets of the cells modulated by either leptin or insulin will greatly influence possible metabolic phenotypes, future studies should consider if these POMC subpopulations are divided not only in responsiveness to selected peptides, but also in anatomical projections and ultimately how this may influence glucose and energy homeostasis.

In conclusion, we recently assessed the effect of suppressing PI3K in POMC neurons and found that PI3K signaling was required for the acute effects of leptin to activate POMC neurons (Hill et al., 2008). Additionally, POMC neuronal inhibition in response to insulin was also PI3K

dependent. Our current study showed both histological and electrophysiological evidence which details an action of leptin and insulin in disparate subgroups of arcuate POMC cells. These data extend and reconcile findings demonstrating that insulin and leptin differentially affects Arc POMC neurons via the same intracellular cascade.

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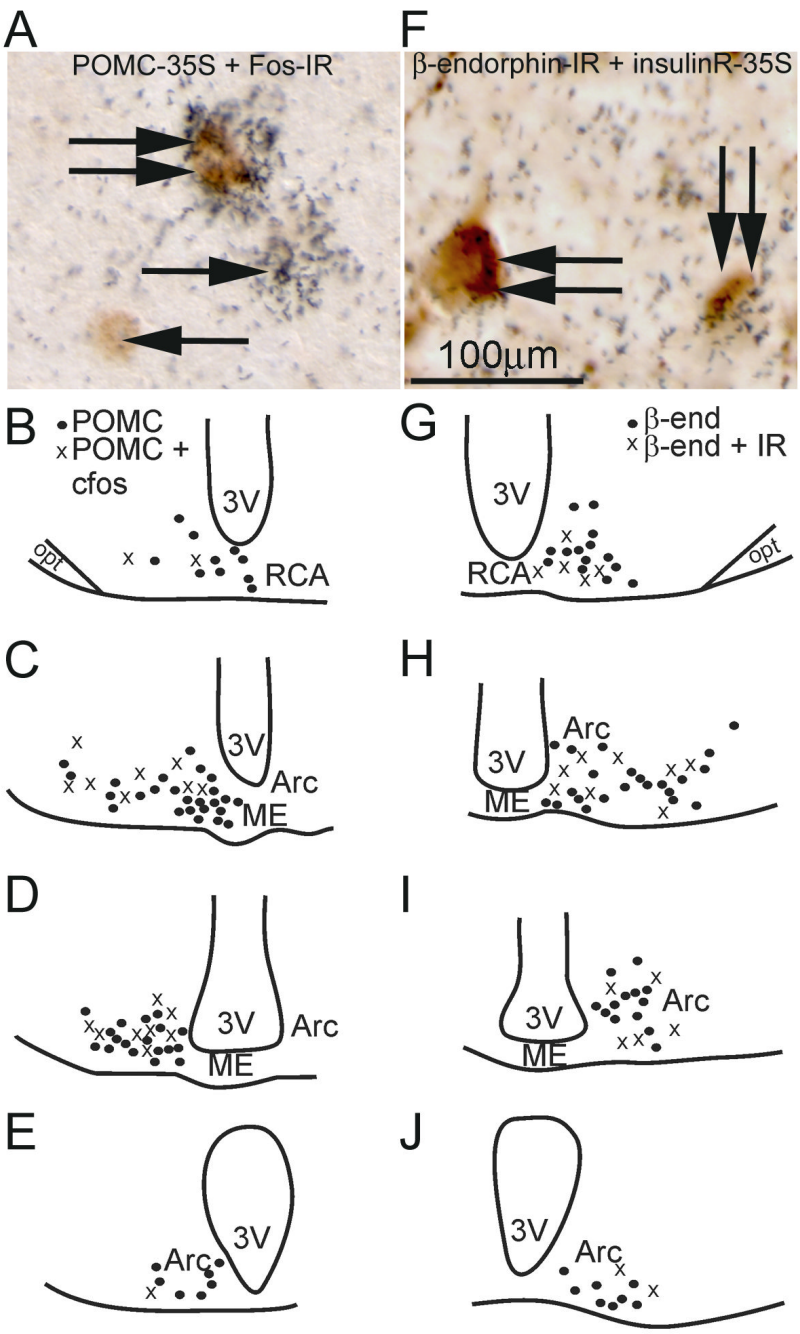


Figure 1. Differential expression of leptin and insulin POMC cells in the retrochiasmatic area and the arcuate nucleus

A. Immunohistochemistry coupled with in situ hybridization histochemistry (ISHH) demonstrates that some POMC neurons express Fos-IR following intraperitoneal leptin. The neurons containing clusters of silver grains were hybridized with 35S-labeled POMC riboprobe. The Fos immunoreactive neurons contain brown nuclear reaction product. Single arrows indicate cells positive for POMC-ISHH or Fos-IR. Double arrows indicate cells positive for both ISHH and immunoreactivity. B-E. Line drawings of four rostral to caudal levels of the mouse hypothalamus illustrate the distribution of Fos-IR POMC neurons in the retrochiasmatic area (RCA) and the arcuate nucleus of the hypothalamus (Arc). F.

Immunohistochemistry coupled with ISHH demonstrates that some B-endorphin containing neurons also express insulin receptor (insulinR). The neurons containing clusters of silver grains were hybridized with 35S-labeled insulinR riboprobe. The B-endorphin immunoreactive neurons contain the brown nuclear reaction product. Double arrows indicate cells positive for both insulinR-ISHH and B endorphin-IR. G-J. Line drawings of four rostral to caudal levels of the mouse hypothalamus demonstrates insulinR expression in B-endorphin neurons in the retrochiasmatic area (RCA) and the arcuate nucleus of the hypothalamus (Arc).

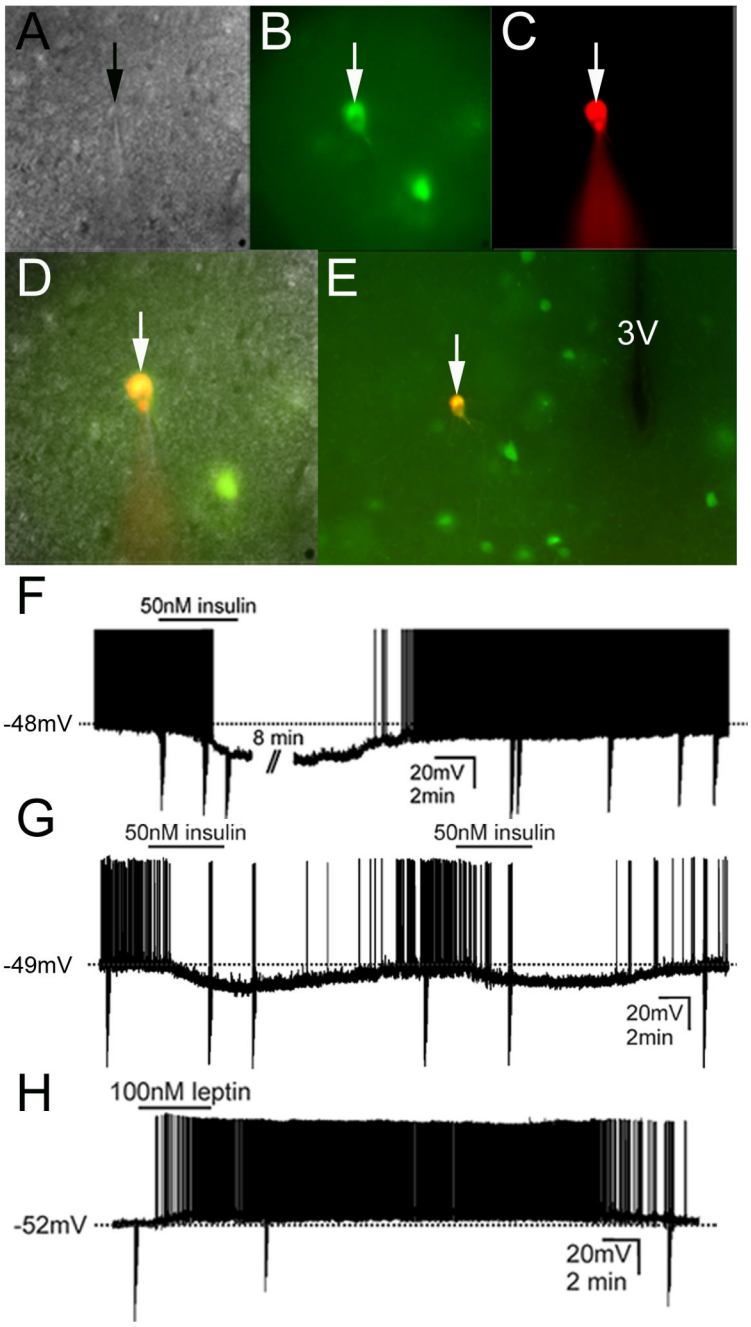


Figure 2. Identification of insulin inhibited and leptin excited POMC neurons in the rectrochiasmatic area and the arcuate nucleus for whole-cell patch-clamp recordings

A. Brightfield illumination of POMC-GFP neuron during acquisition of a whole-cell recording (arrow indicates the targeted cell). **B.** The same neuron under fluorescent (FITC) illumination. **C.** Image shows the complete dialysis of Alexafluor-594 from the intracellular pipette at the end of the recording. **D.** Image illustrates co-localization of Alexafluor-594 with the GFP expression in the same POMC-GFP neuron. **E.** Post-hoc identification of recorded neuron within the RCA in relation to third ventricle (3V). **F.** Current-clamp recording of cell depicted in previous panels (A-E) demonstrating that insulin (50nM) results in hyperpolarization of some POMC-GFP neurons. **G.** Current-clamp recording of a POMC-GFP neuron which

hyperpolarized in response to successive application of insulin (50nM). **H.** Current-clamp recording of a different cell demonstrating that leptin (100nM) results in a depolarization in some POMC-GFP neurons. Traces (F, G, and H) depict a reversal within 20-30 minutes following washout. (Downward deflections represent voltage deflections to hyperpolarizing current injections)

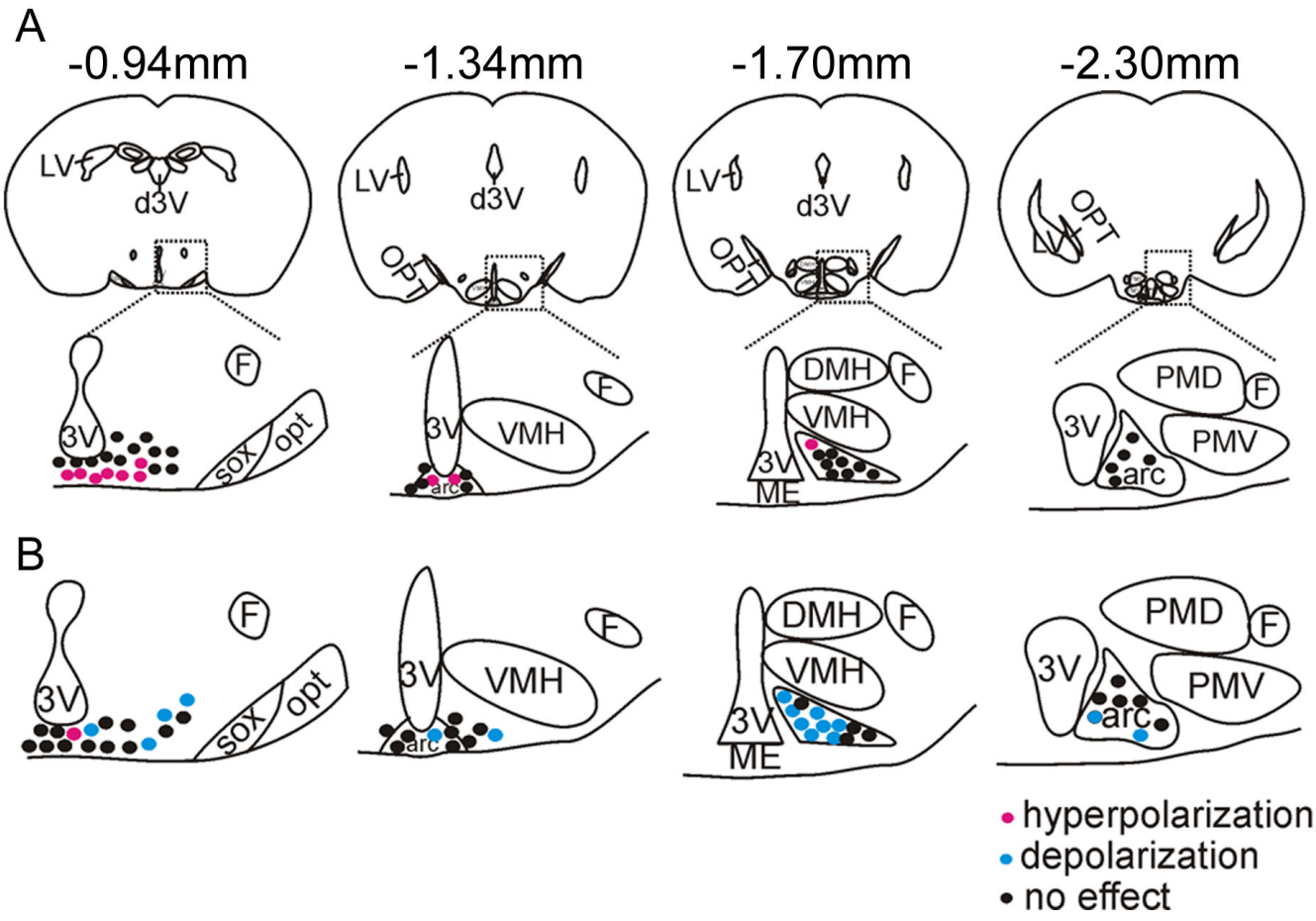
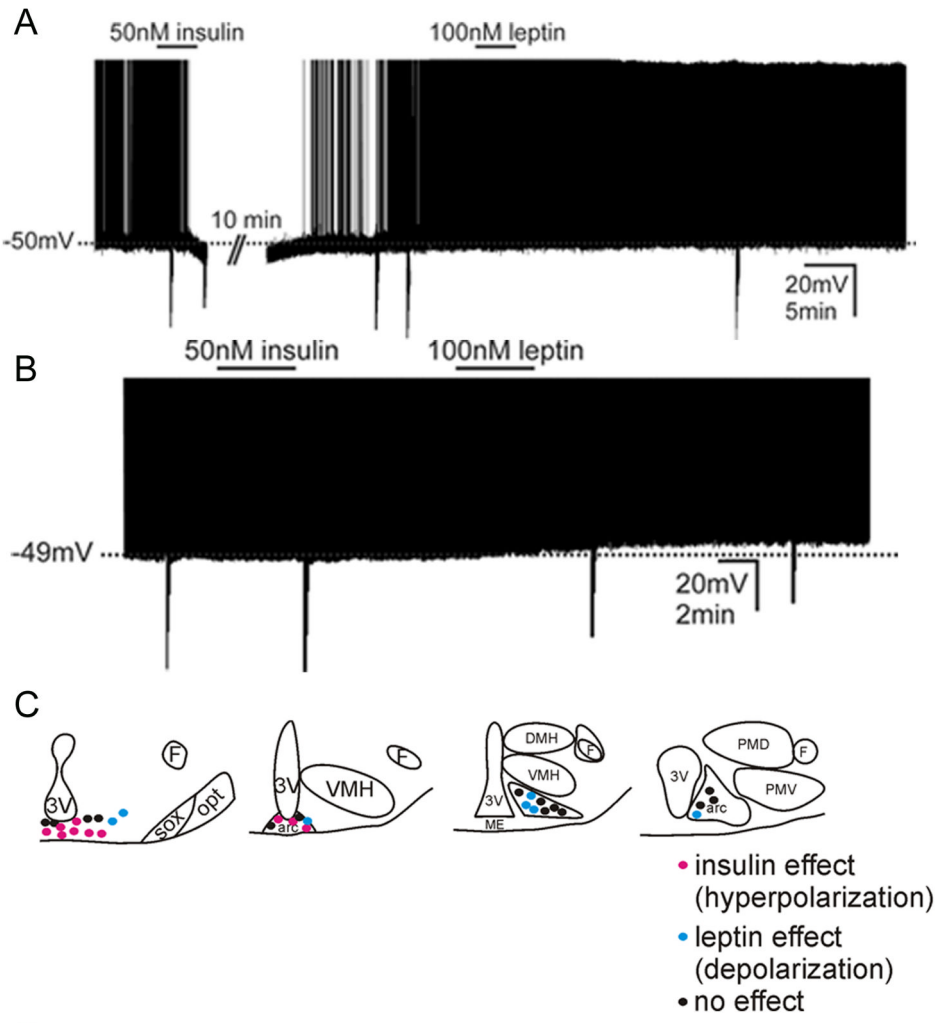


Figure 3. Leptin and insulin acutely modify the cellular activity of POMC-GFP neurons throughout the retrochiasmatic area and the arcuate nucleus

A. Representative drawings at four rostro-caudal levels of the mouse hypothalamus illustrate the location of insulin-inhibited POMC-GFP neurons. B. Drawings at four rostro-caudal levels of the mouse hypothalamus demonstrate the location of leptin-excited POMC-GFP neurons.



Region	Recorded cells	Leptin activated cells	Percent leptin-activated
RCA	13	2	15.4
Arc1	6	1	16.7
Arc2	8	3	37.5
Arc3	4	1	25.0

Region	Insulin inhibited cells	Percent insulin-inhibited	Unresponsive cells	Percent unresponsive
RCA	7	53.8	5	38.5
Arc1	3	50.0	2	33.3
Arc2	0	0.0	5	62.5
Arc3	0	0.0	3	75.0

Figure 4. Leptin and insulin modify the acute cellular activity of disparate POMC-GFP neurons in the retrochiasmatic area and the arcuate nucleus

A. Current-clamp recording shows RCA and arcuate POMC-GFP cells which hypepolarized in response to insulin fail to respond to leptin.

B. Current-clamp trace illustrates RCA and arcuate POMC-GFP cells which depolarized in response to leptin fail to hyperpolarize in response to insulin. (Downward deflections represent voltage deflections to hyperpolarizing current injections) C. Representative map of leptin and insulin responses in the RCA and arcuate nucleus of the hypothalamus. D. Table of responses observed from POMC cells in the presence of insulin followed by leptin.

Table 1

Distribution of Fos-IR and insulinR (IR) in POMC neurons of the retrochiasmatic area and the arcuate nucleus

Values represent estimates of mean counts of cells ± SEM. The mice received an injection of either leptin (n = 5) or PFS (n = 3) intraperitoneally. We used adjacent sections of the same mice for POMC or insulinR in situ hybridization (35S riboprobes). Arc1, arcuate nucleus of the hypothalamus, rostral level; Arc2, arcuate nucleus of the hypothalamus, intermediate level; Arc3, arcuate nucleus of the hypothalamus, caudal level; RCA, retrochiasmatic area.

Region	POMC cells	Double POMC/Fos (saline)	Percent Double POMC/Fos (saline)
RCA	17.0 ± 3.2	1.3 ± 0.3	9.4 ± 4.4
Arcl	37.3 ± 4.3	0.7 ± 0.6	1.7 ± 0.9
Arc2	16.6 ± 2.0	1.0 ± 0.6	5.3 ± 2.9
Arc3	7.3 ± 1.5	0.0 ± 0.0	0.0 ± 0.0
Region	**POMC cells**	**Double POMC/Fos (leptin)**	**Percent Double POMC/Fos (leptin)**
RCA	13.4 ± 1.8	4.4 ± 0.7	33.6 ± 3.0
Arcl	41.0 ± 6.8	10.4 ± 2.0	25.0 ± 4.5
Arc2	22.0 ± 5.5	4.2 ± 0.8	19.3 ± 2.6
Arc3	6.8 ± 3.5	0.2 ± 0.2	3.3 ± 3.3
Region	**β-endorphin cells**	**Double β-endorphin/IR**	**Percent Double β-endorphin/IR**
RCA	15.7 ± 2.0	4.3 ± 0.8	27.9 ± 3.8
Arcl	43.0 ± 3.8	11.3 ± 0.9	26.4 ± 0.6
Arc2	17.0 ± 1.5	6.3 ± 1.5	36.4 ± 4.4
Arc3	5.0 ± 3.0	0.6 ± 0.6	6.1 ± 4.7

Table 2

Distribution of leptin-activated and insulin-inhibited POMC neurons in the retrochiasmatic area and the arcuate nucleus

Values represent the number and percentage of POMC neurons (RCA and Arc) which were targeted and either depolarized or hyperpolarized in the presence of leptin and insulin, or remained unchanged. Arc1, arcuate nucleus of the hypothalamus, rostral level; Arc2, arcuate nucleus of the hypothalamus, intermediate level; Arc3, arcuate nucleus of the hypothalamus, caudal level; RCA, retrochiasmatic area.

Region	Recorded cells	Leptin-activated cells	Percent leptin-activated cells
RCA	17	4	23.5
Arc1	10	2	20.0
Arc2	12	8	66.7
Arc3	7	2	28.6
Region	**Recorded cells**	**Insulin-inhibited cells**	**Percent insulin-inhibited cells**
RCA	19	7	36.8
Arc1	7	2	25.0
Arc2	9	1	11.1
Arc3	5	0	0.0