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Pauline M. Smith and Alastair V. Ferguson

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The subfornical organ: a central nervous system site for actions of circulating leptin

P. M. Smith,^{1*} A. P. Chambers,^{2*} C. J. Price,¹ W. Ho,² C. Hopf,¹ K. A. Sharkey,² and A. V. Ferguson¹

¹Department of Physiology, Queen's University, Kingston, Ontario, Canada; and ²Department of Physiology and Biophysics, Hotchkiss Brain Institute and Snyder Institute of Infection, Inflammation and Immunity, University of Calgary, Calgary, Alberta, Canada

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Smith PM, Chambers AP, Price CJ, Ho W, Hopf C, Sharkey KA, Ferguson AV. The subfornical organ: a central nervous system site for actions of circulating leptin. *Am J Physiol Regul Integr Comp Physiol* 296: R512–R520, 2009. First published November 21, 2008; doi:10.1152/ajpregu.90858.2008.—Adipose tissue plays a critical role in energy homeostasis, secreting adipokines that control feeding, thermogenesis, and neuroendocrine function. Leptin is the prototypic adipokine that acts centrally to signal long-term energy balance. While hypothalamic and brain stem nuclei are well-established sites of action of leptin, we tested the hypothesis that leptin signaling occurs in the subfornical organ (SFO). The SFO is a circumventricular organ (CVO) that lacks the normal blood-brain barrier, is an important site in central autonomic regulation, and has been suggested to have a role in modulating peripheral signals indicating energy status. We report here the presence of mRNA for the signaling form of the leptin receptor in SFO and leptin receptor localization by immunohistochemistry within this CVO. Central administration of leptin resulted in phosphorylation of STAT3 in neurons of SFO. Whole cell current-clamp recordings from dissociated SFO neurons demonstrated that leptin (10 nM) influenced the excitability of 64% (46/72) of SFO neurons. Leptin was found to depolarize the majority of responsive neurons with a mean change in membrane potential of 7.3 ± 0.6 mV (39% of all SFO neurons), while the remaining cells that responded to leptin hyperpolarized (-6.9 ± 0.7 mV, 25% of all SFO neurons). Similar depolarizing and hyperpolarizing effects of leptin were observed in recordings from acutely prepared SFO slice preparations. Leptin was found to influence the same population of SFO neurons influenced by amylin as three of four cells tested for the effects of bath application of both amylin and leptin depolarized to both peptides. These observations identify the SFO as a possible central nervous system location, with direct access to the peripheral circulation, at which leptin may act to influence hypothalamic control of energy homeostasis.

circumventricular organ; hypothalamus

ADIPOSE TISSUE PLAYS A CRITICAL role in energy homeostasis, secreting adipokines that control feeding, thermogenesis, immunity, and neuroendocrine function. Leptin, a 16 kDa peptide, is the prototypic adipokine that has been shown to be an important afferent signal regulating body weight. A missense mutation in the *ob* gene (the gene that encodes leptin) in the *ob/ob* mouse, results in a markedly obese phenotype that is normalized by both systemic and central leptin administration (9, 19, 37). Leptin administration also has been shown to dose-dependently decrease body weight, preferentially reducing body fat while sparing lean tissue in both *ob/ob* and

wild-type mice (18, 19). Plasma leptin levels reflect both energy stores and acute energy balance. Circulating leptin decreases food intake and increases energy expenditure through activation of receptors in hypothalamic and brain stem neurons (16).

The leptin receptor, encoded by the *Ob-R* gene, was isolated from choroid plexus by expression cloning and is a member of the cytokine family (48). Although five leptin receptor isoforms have been identified (Ob-Ra to Ob-Re), only the long form of the receptor, Ob-Rb, possesses the cytoplasmic domains required for signal transduction (1, 5, 27). Ob-Rb regulates multiple intracellular signaling cascades, including the janus-activating kinase signal transducer and activator of transcription pathway and the phosphoinositol-3 kinase and adenosine monophosphate kinase pathways, and is essential for the weight-reducing effect of leptin (4–6). In particular, Ob-Rb is found in hypothalamic nuclei involved in feeding behavior, including the arcuate nucleus (ARC), paraventricular nucleus, dorsomedial nucleus, and the lateral hypothalamic area (31), and it is clear that leptin signaling in these structures plays a pivotal role in regulating energy balance.

The presence of the blood-brain barrier (BBB) leads to the obvious question as to how this peripheral peptide gains access to the central sites. While peptide transporter systems (2) and transendothelial signaling (35) represent mechanisms through which peripheral signals may reach hypothalamic neurons behind the BBB, an alternative explanation also deserves consideration. The sensory circumventricular organs (CVOs) are a group of central nervous system structures that lack the normal BBB. These specialized regions have been shown to contain a dense vasculature, fenestrated epithelium, and the presence of a large variety of peptidergic receptors. Thus, the CVOs are uniquely suited to detect circulating signals and relay this information via well-documented efferent pathways to hypothalamic autonomic nuclei (for a review, see Ref. 15). A role for the sensory CVOs mediating weight-reducing effects of leptin is supported by a recent study demonstrating that the area postrema, a hindbrain CVO, may play a role in mediating a synergistic weight loss effect of amylin and leptin in leptin-resistant diet-induced obesity rats (43). A potential role for the subfornical organ (SFO), a forebrain CVO, in energy homeostasis is suggested by its neural projections to hypothalamic areas with well-documented roles in energy homeostasis and the distribution of a number of different receptors for peripheral signals reflecting the animal's energy status. Elec-

* P. M. Smith and A. P. Chambers contributed equally to this paper.

Address for reprint requests and other correspondence: A. V. Ferguson, Dept of Physiology, Queen's Univ., Kingston, Ontario, Canada K7L 3N6 (e-mail: avf@queensu.ca).

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trophysiological studies have demonstrated that SFO neurons project to the paraventricular nucleus and lateral hypothalamic area (46, 47) and that glutamate stimulation of ARC neurons alters the firing rate of SFO neurons (41). In addition, autoradiographic studies have demonstrated reciprocal SFO connections with the lateral hypothalamic area (33). A suggested involvement for the SFO in the regulation of energy homeostasis is also derived from studies demonstrating receptors in SFO for the gut peptides ghrelin (39), amylin (10, 44), and peptide YY (26). A role for the SFO in modulating circulating signals reflecting energy status has been suggested by studies in which we demonstrated that ghrelin and amylin, circulating signals with opposite effects on feeding behavior, influence the excitability of different subpopulations of SFO neurons.

Ob-R-like immunoreactivity has been demonstrated in the sensory CVOs (SFO, area postrema, organum vasculosum of the lamina terminalis) (30, 39); however, the specific leptin receptor subtypes were not determined. In addition, recent gene array studies from our own laboratory have revealed the presence of the leptin receptor mRNA in the SFO (20). The present study was undertaken to test the hypothesis that leptin signaling occurs in the SFO. We determined whether the signaling form of the leptin receptor (Ob-Rb) is found in SFO, and examined the functional effects of activation of this receptor on SFO neurons.

METHODS

For all experiments, animals were maintained on a 12:12-h light-dark cycle and were provided with food and water ad libitum prior to experimentation, except where indicated. All animal protocols were in accordance with Canadian Council for Animal Care Guidelines and were approved by the Queen's University and the University of Calgary Animal Care Committees.

Leptin Receptor Localization Using RT-PCR

Male Sprague-Dawley rats (100–150 g; Charles River, St. Constant, Quebec, Canada) were decapitated, and the brains were quickly removed and placed in oxygenated ice-cold artificial cerebrospinal fluid containing (in mM) 124 NaCl, 2 KCl, 1.25 KH₂PO₄, 2.0 CaCl₂, 1.3 MgSO₄, 20 NaHCO₃, and 10 glucose. The SFO was visually identified at the dorsal surface of the third ventricle by using a dissecting microscope and was gently microdissected away from the immediately adjacent hippocampal commissure. Total RNA was extracted from SFO from two rats using an Ambion RNAqueous kit and then were DNase treated (Fermentas) by adding a mixture of 1 μ l 10 \times buffer with MgCl₂, 7 μ l diethylpyrocarbonate-treated H₂O, and 1 μ l deoxyribonuclease to the total RNA, and incubating the solution at 37°C for 30 min. After incubation, 1 μ l of 25 mM of EDTA was added to the solution and incubated at 65°C for 10 min. Oligo(dT)-

based cDNA was synthesized using Superscript III reverse transcriptase kit (Invitrogen, Carlsbad, CA) to make a final reaction volume of 10 μ l.

Two microliters of SFO cDNA were added to a PCR reaction containing: 25 μ l of 2 \times QIAGEN Multiplex PCR Master Mix, 5 μ l of Q solution, 2 μ l of each primer set, and 16 μ l diethyl pyrocarbonate-treated H₂O to a final volume of 50 μ l. To prevent the formation of misprimed products and primer dimers, the HotStarTaq DNA polymerase (contained in the QIAGEN Multiplex PCR Master Mix) was activated by a 15-min, 95°C-incubation step. The reaction tube was then cycled 45 times through a protocol of 94°C for 60 s, 60°C for 60 s, 72°C for 60 s, and finally, 72°C for 10 min. Primers (Integrated DNA Technologies) directed toward an extracellular domain common to all leptin receptor isoforms were used to detect the presence of leptin receptor (LepR) mRNA (see Table 1). To determine whether the signaling form of the receptor (Ob-Rb) mRNA was present in SFO, one previously verified primer set (7) and one that we designed, both of which were specific for the intracellular signaling domains on Ob-Rb were used (See Table 1). Sets of primers were also used to detect Ob-Ra mRNA (see Table 1), as well as GAPDH (+ control). All of the aforementioned primers were also used in hypothalamic tissue, prepared in the same manner as the SFO, which served as a positive tissue control for the presence of LepR, Ob-Rb, and Ob-Ra mRNA. An RT(-) reaction, in which the reverse transcriptase enzyme was omitted from the RT-PCR reaction, was used as a negative control. PCR products were run and visualized on electrophoresis gel containing 2% agarose and ethidium bromide.

Immunohistochemistry

Frozen brain sections 35 μ m thick were cut using a Leica CM3050 S cryostat (Richmond Hill, ON, Canada). For double labeling, sections were rinsed with 0.1% Triton X-100 in PBS, placed in 10% goat serum for 1 h and incubated in primary antisera directed all forms of the leptin receptor of LepR (1:50; cat. no. SC 1834; Santa Cruz Biotechnologies, Santa Cruz, CA), the neuronal marker NeuN (1:100; cat. no. MAB 377; Chemicon, Billerica, MA), or the astrocyte marker glial fibrillary acidic protein (1:250; cat. no. BT-575; Biomedical Technologies, Stoughton, MA) for 24 h. Specificity for the leptin receptor antibody was confirmed by preincubating with the peptide (SC 1834P), the antibody it was raised against, which completely abolished the labeling. Donkey anti-goat FITC (1:100, Jackson ImmunoResearch, West Grove, PA), goat anti-mouse FITC (1:50; Jackson ImmunoResearch), and donkey anti-mouse CY3 (1:100; Biotin Scientific, Mississauga, ON, Canada) were used as secondary antisera. Staining was visualized using a Zeiss Axioplan fluorescence microscope and photographed with a digital camera or an Olympus Fluoview FV300 confocal microscope using krypton-argon and helium neon lasers. Differential visualization of the fluorophores FITC (excitation 490 nm and emission 520 nm) and CY3 (excitation 552 nm and emission 565 nm) was accomplished with the use of specific filter combinations. Samples were scanned sequentially and images were obtained under identical exposure conditions (pinhole aperture, laser

Table 1. Primer sets used in the detection of mRNA from subfornical organ

Gene	Primer	Position	Sequence	Product Size, bp	Accession No.
Glyceraldehyde-3-phosphate Dehydrogenase	GAPDH	Sense	taccaggetgccttctct	469	NM_017008
		Antisense	ctcgtggttcacaccctc		
Leptin receptor Isoform b	OB-Rb1	Sense	gattccacaagggttctc	510	NM_012596
		Antisense	ctggaggattctgatgtc		
Leptin receptor Isoform b	OB-Rb2	Sense	tgaccactccagattccaca	501	NM_012596
		Antisense	tgaacagacagtgagctggg		
Leptin receptor Isoform a	OB-Ra	Sense	acactgttaatttcacaccagag	228	AF304191
		Antisense	agtcattcaaacatagtttagg		
Leptin receptor	LepR	Sense	tgtctctacatcagagctca	140	NM_012596
		Antisense	gtggagagtcaggtaacct		

strength, scan speed, Kalman averaging 2 \times). Confocal images are digital composites of Z-stacks scans 1 μ m thick as detailed in the figure legends. Micrographs were generated with Fluoview Software and CorelDraw.

Leptin-Induced pSTAT3 Signaling

Under ketamine/xylazine (85:15) anesthesia, male Sprague-Dawley rats (250–280 g) were placed in a stereotaxic frame. The skull was exposed, and a 25-gauge stainless steel guide cannula was positioned just above a lateral ventricle according to the coordinates of Paxinos and Watson (bregma; posterior, -1; lateral, 1; ventral, 3.2) (36) using a dental drill. The cannulae were fixed in place with dental acrylic and anchored to the skull with two screws. After 7 days of recovery and an overnight fast, rats were gently restrained and treated with either leptin (cat. no. L5037; Sigma-Aldrich, Oakville, ON, Canada) (5 μ g icv; $n = 3$) or vehicle (PBS, pH 7.4) (5 μ l; $n = 3$) by gravity flow using a 27-gauge needle connected to 20 cm of polyurethane tubing. Rats were returned to the home cage and anesthetized 30 min later (65 mg/kg pentobarbital sodium, Somnotol; MTC Pharmaceuticals, Cambridge, ON, Canada). They were then intracardially perfused with saline followed by ice-cold fixative (1 l/kg 4% paraformaldehyde pH 7.4). The brain from each animal was postfixed overnight at 4°C, washed three times in PBS, and transferred to a 30% sucrose + PBS solution for an additional 24 h. For pSTAT3 immunohistochemistry, sections (prepared as above) were rinsed in PBS and then incubated in 1% NaOH and 1% H₂O₂ for 20 min, 0.3% glycine for 10 min, and 0.6% sodium dodecyl sulfate for 10 min. There were 3 \times 10-min rinses in PBS between each treatment. Sections were blocked in 10% goat serum and placed in anti-pSTAT3 (tyr705) antibody (1:1,000; cat. no. 9131s; Cell Signaling Technology, Danvers, MA) on an orbital shaker at 4°C for 24 h. Sections were rinsed and incubated in a secondary donkey anti-rabbit CY3 (1:100; Biocan Scientific, Mississauga, ON, Canada) antibody for 1 h before being mounted onto glass slides and coverslipped with bicarbonate-buffered glycerol. Cannula placement and evidence that the lateral ventricle had been punctured was confirmed during processing for immunohistochemistry in all six rats.

Electrophysiology

Cell dissociation and short-term primary culture. SFO tissue was acutely dissected from Male Sprague-Dawley rats (100–150 g; Charles River, QC, Canada) as described above. The SFO was then incubated in 5 ml of Hibernate media (Brain Bits, Springfield, IL) containing 2 mg/ml papain (Worthington Biochemical, Lakewood NJ) at 30°C for 30 min. Following incubation, cells were washed, gently triturated in Hibernate media supplemented with B27 (GIBCO-Invitrogen, Burlington, Canada) to liberate single cells, and centrifuged at 400 g for 4 min at 4°C. The supernatant was removed, and the pellet resuspended in B27 supplemented Neurobasal A media (GIBCO-Invitrogen) containing 100 U/ml penicillin/streptomycin and 0.5 mM L-glutamine (GIBCO-Invitrogen). Cells were plated on 35-mm uncoated glass bottom culture dishes (MatTek, Ashland, MA) and placed in a CO₂ incubator (95% O₂-5% CO₂ at 37°C). Additional B27-supplemented Neurobasal A was added to the culture dishes 1 h after plating (to allow adequate time required for cells to adhere to the bottom of the culture dish). Cells were maintained in the incubator until the time of experimentation (1–4 days).

SFO slice preparation and current-clamp electrophysiology. Male Sprague-Dawley rats (Charles River) aged postnatal days 23–27 (~50–100 g) were quickly decapitated. The brain was removed and placed into ice-cold slicing solution (bubbled with 95% O₂-5% CO₂), consisting of (in mM): 87 NaCl, 2.5 KCl, 25 NaHCO₃, 0.5 CaCl₂, 7 MgCl₂, 1.25 NaH₂PO₄, 25 glucose, and 75 sucrose. A tissue block containing the SFO was obtained, and 300- μ m coronal slices were cut using a vibratome. Slices were then incubated in a water bath at 32°C for at least 1 h before recordings commenced in oxygenated artificial

cerebrospinal fluid composed of (in mM): 126 NaCl, 2.5 KCl, 26 NaHCO₃, 2 CaCl₂, 2 MgCl₂, 1.25 NaH₂PO₄, and 10 glucose. Slices were placed in a chamber that was continuously perfused at ~2 ml/min with 28–32°C artificial cerebrospinal fluid. Neurons were visualized using an infrared differential interference contrast system on an upright microscope (Nikon, Japan).

Current-clamp electrophysiology. Whole cell current-clamp recordings from SFO neurons were acquired using an Axopatch 700B patch-clamp amplifier (Molecular Devices, Palo Alto, CA). Stimulation and recording parameters were controlled by Spike2 (version 5) and Signal (version 3) software (Cambridge Electronics Design, Cambridge, UK). Data were filtered at 1 kHz, acquired at 5 kHz, and digitized using a Cambridge Electronics Design Micro1401 interface. Capacitive transients and series resistance errors were minimized before recording. For all recordings, the external recording solution contained the following (in mM): 140 NaCl, 5 KCl, 1 MgCl₂, 2 CaCl₂,

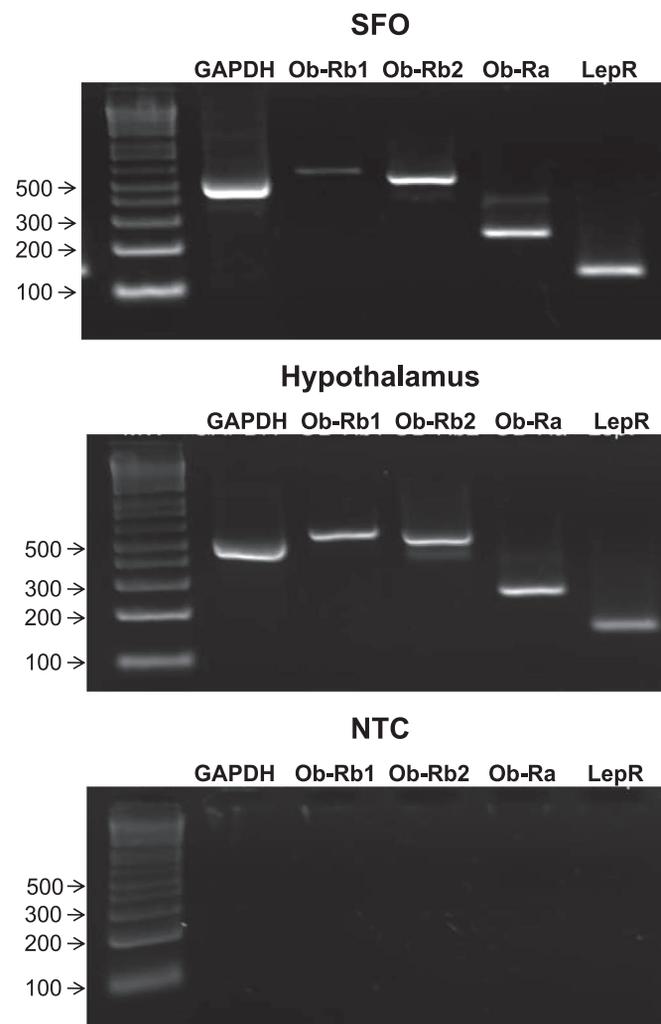


Fig. 1. Ob-Rb receptor mRNA is expressed in the subfornical organ (SFO). Agarose gels showing RT-PCR analysis of SFO cDNA for leptin receptor expression (*top*). Ob-Rb receptor mRNA (Ob-Rb1, Ob-Rb2), as well as Ob-Ra receptor mRNA (Ob-Ra), and leptin receptor (LepR) mRNA (expression common to all leptin receptor isoforms) were also expressed in the SFO. The hypothalamus (*middle*), a positive control tissue, also shows Ob-Rb receptor (Ob-Rb1, Ob-Rb2), Ob-Ra (Ob-Ra), and LepR expression. A primer set specific for GAPDH served as a positive control in both SFO and hypothalamic tissue. PCR products for GAPDH and all leptin receptors (Ob-Rb1, Ob-Rb2, Ob-Ra, and LepR) are not observed in the no template control (NTC) lane in which the template has been omitted from the cDNA synthesis reaction. Product size is (base pairs) shown in the leftmost lane of each gel.

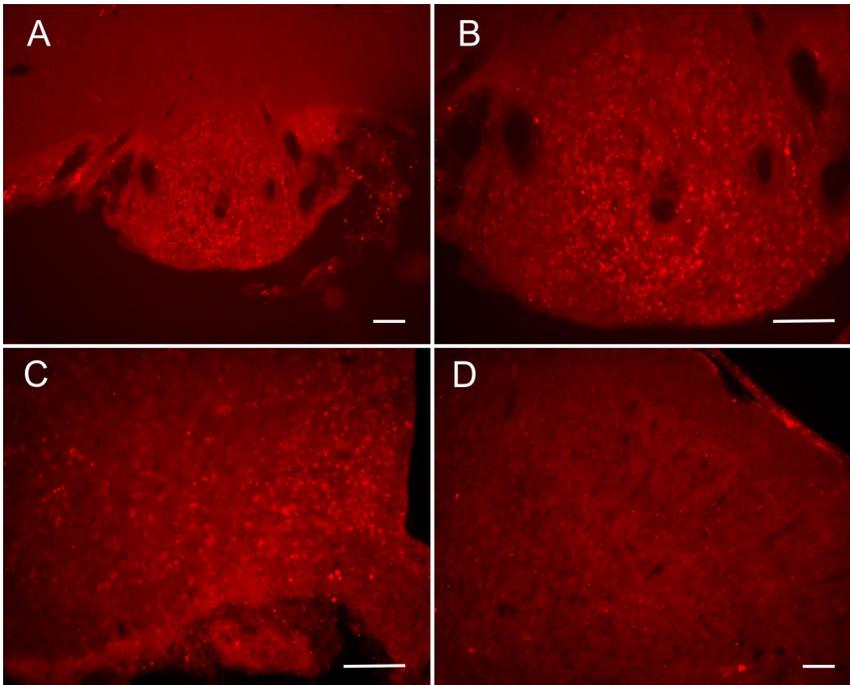


Fig. 2. Leptin receptor is expressed on neurons in the SFO. Immunofluorescence micrographs of leptin receptor immunoreactivity in the SFO (A and B), arcuate nucleus (C), and frontal cortex (D). Leptin receptor immunoreactivity was detected in the SFO and, as expected, in the arcuate nucleus (bregma, -2.8 mm), which served as a positive control. No labeling was observed in the frontal cortex at the same level as the SFO (bregma, -0.8 mm). Scale bars = 100 μ m.

10 HEPES, and 10 glucose, pH 7.3 with NaOH. Patch electrodes were made from borosilicate glass (World Precision Instruments, Sarasota, FL) on a Flaming Brown micropipette puller (model P87; Sutter Instrument, Novato, CA). Electrodes were then fire polished and had resistances of 2.5 – 5 $M\Omega$ when filled with internal recording solution that contained (in mM) 130 K-gluconate, 10 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, 10 EGTA, 4 Na_2ATP , and 0.1 GTP. All chemicals were purchased from Sigma (Oakville, ON, Canada).

Once whole cell configuration was achieved, cells were perfused via a gravity-fed perfusion system with external recording solution at a rate of 2 ml/min. Cells were defined as neurons by the presence of ≥ 50 mV action potentials. Following a minimum 5-min stable baseline recording period (control), 10 nM leptin (rat, recombinant; Phoenix Pharmaceuticals, Belmont, CA; reconstituted in external recording solution) was bath applied (leptin) followed by a wash with external recording solution (wash). Responsiveness of SFO neurons

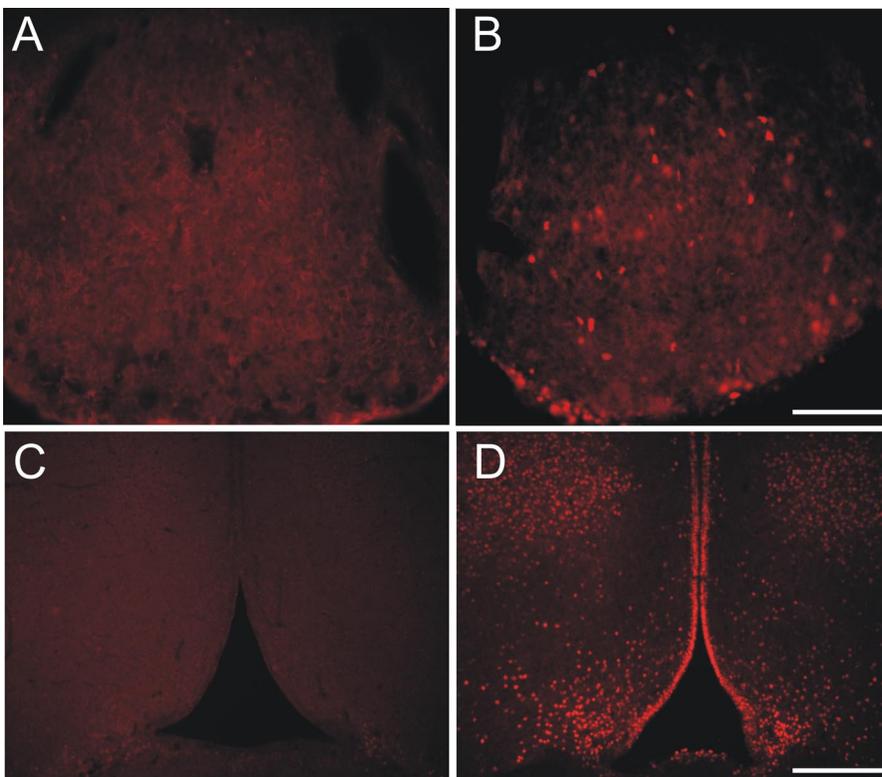


Fig. 3. Leptin induces pSTAT3 activation in the SFO. Immunofluorescence micrographs of pSTAT3 immunoreactivity in the SFO (A and B) and hypothalamus (C and D) of a vehicle (A and C) and leptin-treated rat (B and D). pSTAT3 immunoreactivity was not detected in animals injected intracerebroventricularly with saline. Note that pSTAT3 immunoreactivity was observed in the ventromedial and arcuate nuclei in the leptin-treated animal (bregma, -3.4 mm). Scale bars = 100 μ m.

was determined by comparing membrane potential of neurons before and after leptin perfusion. SFO neurons were considered responsive if the mean membrane potential demonstrated a shift of at least 3 mV (over a 100-s period) during the 300 s following the initiation of bath perfusion with leptin compared with the 100 s before application and demonstrated a partial recovery to baseline values following removal of the peptide from the bath (wash). Alternatively, in the few cells exhibiting regular high-frequency spontaneous action potentials (>4 Hz), a cell was considered responsive if it exhibited a change of at least 1 Hz (in a 100-s period) during the 300 s following the initiation of bath perfusion with leptin. To determine whether leptin acts on amylin-sensitive SFO neurons, the effect of bath administration of amylin (10 nM) and leptin (10 nM) was evaluated on the same SFO neurons.

Statistics

Mean change (means \pm SE) in membrane potential before (control) and during leptin peptide administration (leptin) were calculated, and differences were tested using a paired Student's *t*-test. All statistical analyses were performed using GraphPad Prism (version 5.0; San Diego, CA).

RESULTS

Leptin receptor (Ob-Rb) Expression in SFO

Using primer sets directed toward an extracellular domain common to all leptin receptor isoforms (RT-PCR reactions performed on cDNA obtained from mRNA isolated from acutely microdissected SFO) revealed the presence of leptin

receptor (LepR) mRNA (Fig. 1). To determine whether the signaling form of the leptin receptor Ob-Rb was present in SFO, two different primer sets, each directed toward unique intracellular signaling domains on the Ob-Rb receptor were used, and the data presented in Fig. 1 demonstrate the presence of Ob-Rb mRNA (Ob-Rb1, Ob-Rb2) in SFO. Ob-Ra mRNA was also present in SFO as illustrated in Fig. 1. RT-PCR reactions were also performed on cDNA from acutely microdissected hypothalamus, an area of the brain previously shown to express Ob-Rb (14), which served as a positive tissue control (Fig. 1). LepR, Ob-Rb, and Ob-Ra mRNA were found in all samples of hypothalamus where appropriate-sized products were localized. GAPDH served as a positive control for the PCR reactions. No labeling was found in the negative controls. The validity of all PCR products was confirmed by sequencing.

The presence of the leptin receptor on SFO neurons was confirmed using immunohistochemistry with antibodies raised to an epitope between amino acids 850 and 900, thereby recognizing all isoforms of the leptin receptor. Using this antibody, clear immunoreactivity was observed in the SFO, in addition to the predicted immunoreactivity in the arcuate nucleus, while the lack of immunoreactivity in the frontal cortex, a region known not to express leptin receptors, further supports the specificity of this antibody (Fig. 2). Double labeling showed immunoreactivity for the Ob-Rb on neurons labeled with the neuronal nuclear marker NeuN, while Ob-Rb immu-

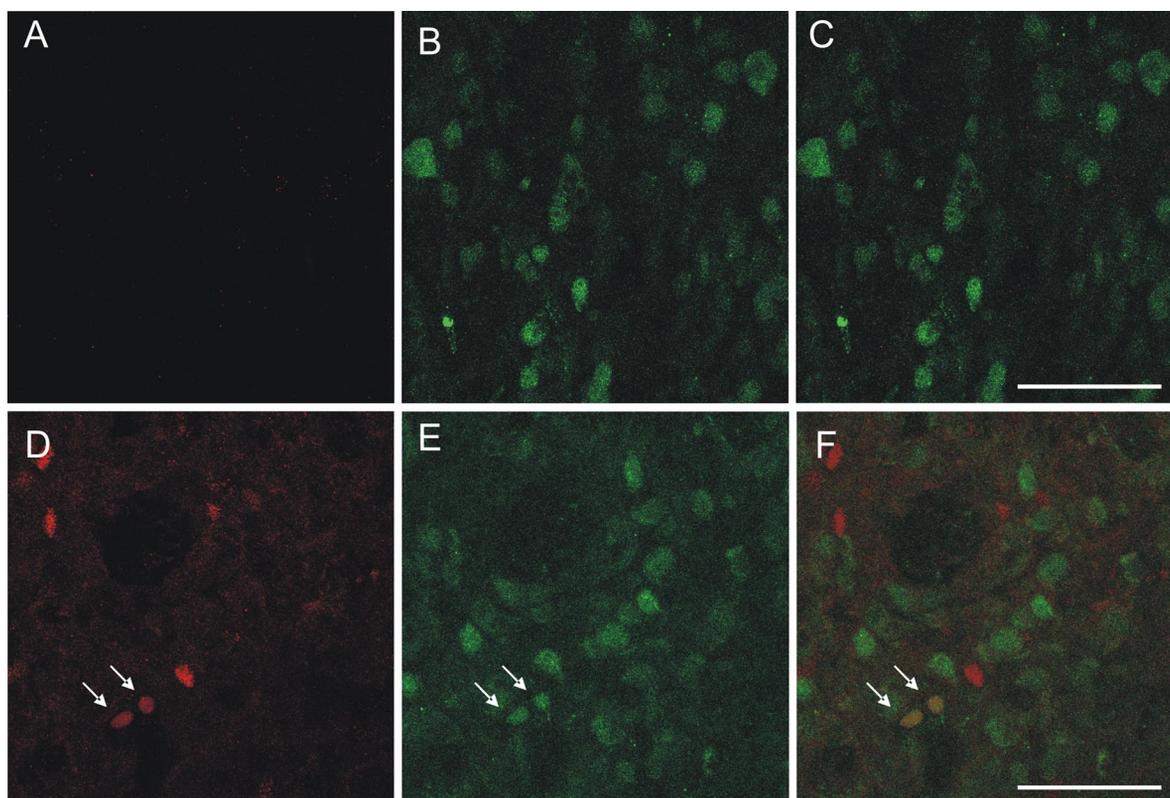


Fig. 4. pSTAT3 is colocalized with the neuronal marker NeuN in the SFO. Confocal immunofluorescence micrographs of pSTAT3 (A and D) and NeuN (B and E) immunoreactivity in the SFO of a vehicle (A–C) and leptin treated rat (D–F). Micrographs of the SFO of a vehicle-treated rat illustrate no pSTAT3 immunoreactivity (A) in neurons whose nuclei are labeled with NeuN (B). C is an overlay of the two images (26, 1- μ m optical sections). Micrographs of the SFO of a leptin-treated rat illustrate pSTAT3 immunoreactivity (D) in neurons whose nuclei are labeled with NeuN (E). F is an overlay of the two images (13, 1- μ m optical sections). Arrows indicate where pSTAT3 and NeuN colocalize. Note that in the SFO NeuN is relatively weakly immunoreactive compared with other regions of the brain. Scale bars = 50 μ m.

noreactivity also appeared to be coexpressed on some glial processes that double-labeled with an antibody directed against glial fibrillary acidic protein, albeit to a much lesser degree than with the neuronal marker NeuN (data not shown).

Leptin Receptor Signaling in SFO

We next examined whether central administration (lateral ventricle) of leptin induced leptin receptor signaling in the SFO by using pSTAT3 immunoreactivity (21, 23, 24). These experiments demonstrated that leptin induced pSTAT3 immunoreactivity in the SFO and hypothalamus of leptin-treated rats (Fig. 3, B and D). To verify that pSTAT3 activation was a consequence of leptin administration and not due to inflammatory effects of cannulation and microinjection procedures, the effects of PBS (vehicle) injections into the lateral ventricle were also examined, and, as can be seen in Fig. 3, these injections did not induce pSTAT3 immunoreactivity in the SFO or hypothalamus (Fig. 3, A and D). The pattern of staining in the hypothalamus was consistent with what has been reported by others (28), with extensive activation in the ventral medial and arcuate nuclei. Neuronal localization of pSTAT3 immunoreactivity in SFO neurons was confirmed using the neuronal-specific marker NeuN as illustrated in Fig. 4.

Electrophysiology

Dissociated SFO neurons. Whole cell current-clamp techniques were used to evaluate the direct effects of leptin receptor activation on dissociated SFO neurons. The dissociation process leaves us with single SFO cells in synaptic isolation (no visible dendritic contacts), which are thus ideally suited for the assessment of direct effects of exogenously applied leptin (10 nM) on the excitability of SFO. Cells were classified as neurons if they exhibited spontaneous action potentials or if application of a short (100 ms) depolarizing current pulse evoked action potentials. SFO neurons were required to demonstrate action potentials (spontaneous or evoked) of at least 50 mV and stable resting membrane potentials to be considered "healthy" and tested for the effects of leptin. In accordance with these criteria, current-clamp recordings were obtained from 72 SFO neurons. Of these neurons, the majority (85%, 61/72 cells) were spontaneously active. The remaining cells were quiescent but fired action potentials in response to a brief depolarizing current pulse. The mean resting membrane potential was -48.8 ± 1.2 mV for all cells obtained. There was no difference in resting membrane potential between those cells that were spontaneously active (mean resting membrane po-

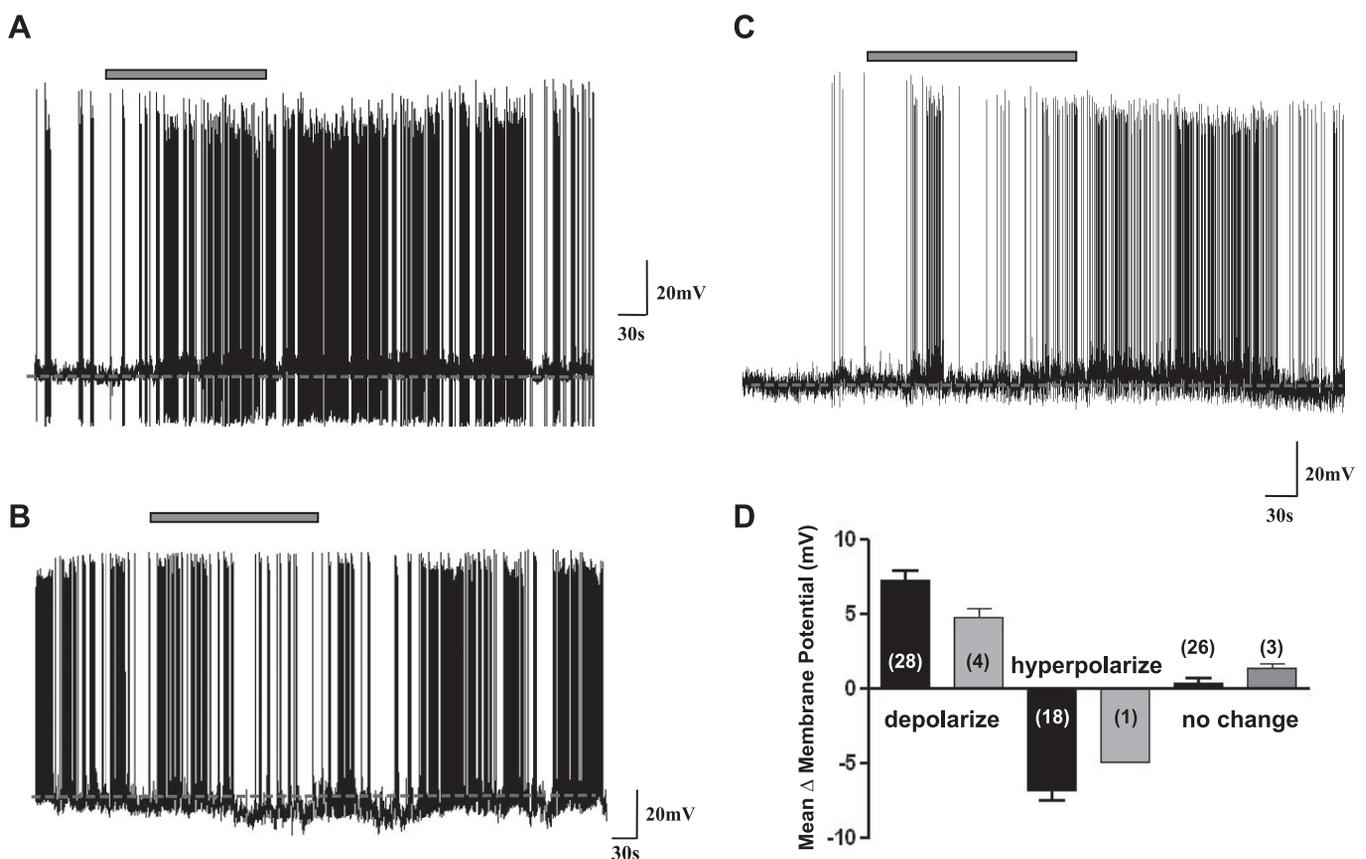


Fig. 5. Leptin influences the excitability of SFO neurons. *A*: current-clamp recording from a spontaneously active dissociated SFO neuron showing that bath application of 10 nM leptin caused a depolarization accompanied by an increase in action potential frequency. *B*: current-clamp recording from a different dissociated SFO neuron that exhibited a hyperpolarizing response and a decrease in action potential frequency in response to bath application of 10 nM leptin. In both neurons, a return to baseline membrane potential and frequency after removal of leptin from the bath was seen. *C*: current-clamp recording obtained from a spontaneously active SFO neuron in a slice preparation illustrating a depolarizing response to 10 nM leptin. *D*: bar graph summarizing the effects of leptin on membrane potential of dissociated SFO neurons (black bars) and SFO neurons obtained from a slice preparation (grey bars) illustrating similar effects on membrane excitability. Numbers in brackets indicate number of cells tested. Time and duration of leptin administration is indicated by the gray bar above each current-clamp recording.

tential = -48.9 ± 1.7 mV) and quiescent cells (mean resting membrane potential = -47.7 ± 3.0 mV, $P = .72$).

Bath application of leptin (10 nM) influenced 64% (46/72) of SFO neurons tested. The majority of responsive neurons (28/46) exhibited a depolarization in response to leptin administration. All depolarizing responses began within 100 s of leptin application, with many cells beginning their depolarizing response within 30 s of bath perfusion with leptin. The depolarizing responses seen in response to leptin administration were of a long duration, lasting several minutes upon termination of leptin application. In fact, only half of the depolarizing effects were completely reversible, showing a recovery to baseline membrane potentials during the period of the recording (see Fig. 5, *top trace*). The mean change in membrane potential of these depolarizing cells was 7.3 ± 0.6 mV ($n = 28$) and was typically accompanied by an expected increase in action potential firing frequency. The remaining affected cells (18/46) demonstrated hyperpolarizing effects in response to leptin administration. Similar to the depolarizing responses, these effects began within 100 s of peptide administration, lasted several minutes, and were reversible in 50% of neurons (see Fig. 5, *bottom trace*). The mean change in membrane potential of hyperpolarizing SFO neurons was -6.9 ± 0.8 mV ($n = 18$). In spontaneously active neurons, these hyperpolarizing effects were accompanied with a decrease in action potential firing frequency. To ensure that leptin responsiveness of SFO neurons was not the result of transformation of these cells following dissociation, the effect of leptin on SFO neurons in slice preparations were also evaluated. Whole cell current-clamp recordings from eight SFO neurons in SFO slices showed similar responsiveness to bath application of 10 nM leptin with 50% of cells depolarized (mean change in membrane potential 4.8 ± 0.6 mV, $n = 4$, Fig. 5), one cell hyperpolarized (-4.9 mV), and the remaining three cells tested being unaffected by peptide application.

To determine whether leptin influenced the same population of SFO neurons influenced by amylin, which depolarizes SFO neurons (38), 11 cells were tested for the effects of bath administration of both leptin and amylin. Three of four cells that depolarized to leptin also depolarized to amylin (see Fig. 6). Cells that hyperpolarized in response to leptin ($n = 3$) were not influenced by bath application of amylin, while the remaining four cells were not influenced by administration of either peptide.

DISCUSSION

In this study, we demonstrate the presence of the signaling form of the leptin receptor in the SFO and the responsiveness of SFO neurons to leptin. Using RT-PCR technology, we have, for the first time, demonstrated the presence of the signaling form of the leptin receptor Ob-Rb mRNA as well as the presence of Ob-Ra mRNA in acutely dissected whole SFO. The same primers also detected Ob-Rb mRNA and Ob-Ra mRNA in acutely dissected hypothalamus, an area known to contain both receptor subtypes (for a review, see Ref. 25). These results confirm and extend recent findings of gene array studies from our laboratory, demonstrating the presence of leptin receptor mRNA in the SFO (20). In the present study, we sought to identify whether the mRNA was translated into a functional receptor using two approaches: 1) immunohistochemistry for the receptor and 2) leptin-induced activation of

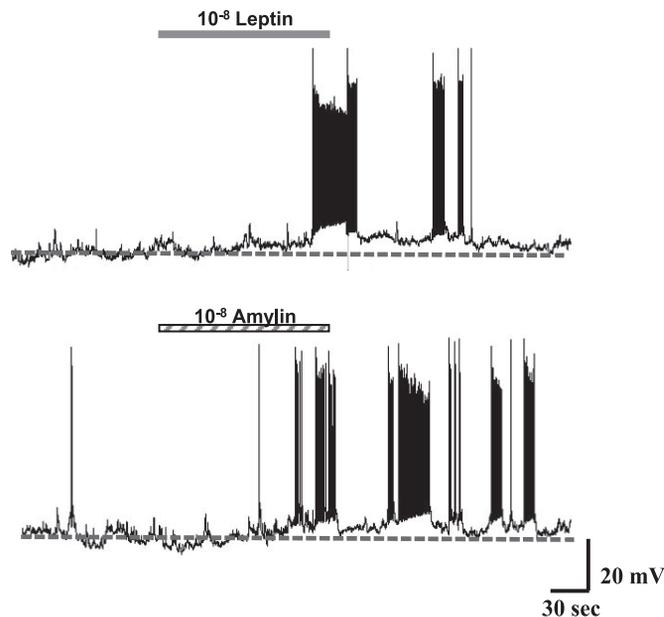


Fig. 6. Leptin depolarizes amylin-sensitive SFO neurons. Current-clamp recording obtained from the same SFO neuron illustrating depolarizing responses to leptin (*top trace*) and amylin (*bottom trace*). Time and duration of leptin (*top trace*, solid bar) and amylin (*bottom trace*, hatched bar) administration is indicated by the bar above each current-clamp recording.

STAT3, revealed as nuclear labeling of pSTAT3 immunoreactivity. We observed immunoreactivity in the SFO of rats by using an antibody raised to an epitope between amino acids 850 and 900 (thus identifying all leptin receptor isoforms), confirming the presence of the leptin receptor in SFO. The neuronal identity of some of these cells was confirmed using the neuronal marker NeuN. Leptin receptor immunoreactivity was also coexpressed on a small population of glial processes in the SFO, suggesting potential roles for leptin in influencing both neurons and glial cells. These findings were not completely unexpected, as previous studies have shown that OB-Rb is expressed in both neurons (8, 17, 32) and primary glial cell cultures (22). We are aware that the specificity of widely used antibodies directed against the leptin receptor has been a point of controversy in the field of obesity research (34). While some labeling using antibodies is certainly “real,” there are some sites in the brain where immunoreactivity is observed in the absence of the mRNA identified by *in situ* hybridization. In view of this issue, we chose not to rely exclusively on this approach to identify functional leptin receptor expression in SFO. In our studies, we also examined the localization of pSTAT3, a well-accepted marker of Ob-Rb receptor activation as an additional indicator of the presence of functional Ob-Rb in SFO. Central and peripheral leptin administration has been shown to increase STAT3 phosphorylation in hypothalamic and brain stem nuclei involved in the regulation of feeding (21) and that pSTAT3 activation is necessary for the inhibitory effect of leptin on food intake and body weight (38). In the present study, we have shown that leptin induced pSTAT3 expression in the SFO (as well as in the hypothalamus, as previously described), while little or no expression was observed in vehicle-treated animals, indicating that pSTAT3 activation was specific to leptin treatment and not due to

inflammatory processes that may have arisen as a consequence of cannula placement/microinjection into the ventricle.

Having shown that leptin activates pSTAT3 in SFO neurons, we wanted to explore whether there were short-term signaling consequences of leptin receptor activation in this organ. The results of our current-clamp experiments clearly demonstrate that leptin influences the excitability of the majority (64%) of dissociated SFO neurons tested. The effects of leptin on dissociated neurons are the result of direct actions of leptin, as the process of dissociation isolates single SFO neurons rendering them devoid of any synaptic input. Our findings that similar effects were seen in slice preparations confirms that such responsiveness of SFO neurons is not a consequence of transformation of these cells following dissociation. The presence of subpopulations of neurons in the SFO may explain the finding of both depolarizing and hyperpolarizing responses to leptin administration. Previous studies, from our laboratory and others, have demonstrated heterogeneous responses of SFO neurons (depolarizing and hyperpolarizing) to a variety of peptidergic substances (11). Although not addressed in the present study, perhaps the heterogeneity in excitability of SFO neurons in response to leptin administration reflects the existence of different subpopulations of SFO neurons with specific, yet different hypothalamic projection sites, which together contribute to the coordinated effects of leptin on food intake.

Evidence in support of a potential role for the SFO in the control of energy balance is emerging from a number of different observations. Neurons in the SFO have been shown to be responsive to both the appetite-suppressing hormone amylin (3, 40) and the appetite-stimulating hormone ghrelin (39), with this recent study from our own laboratory showing that different populations of SFO neurons are depolarized by either amylin or ghrelin, circulating signals that have opposite effects on food intake, with no SFO cells responding to both peptides. The findings of the present study, demonstrating depolarizing and hyperpolarizing effects of leptin on different SFO neurons, also support the existence of subpopulations of SFO neurons and would suggest that neurons that depolarize to leptin may be those that are similarly influenced by the anorexigenic peptide amylin. To test this hypothesis, we evaluated the effects of bath application of both amylin and leptin on individual SFO neurons and have shown that leptin depolarizes amylin-responsive SFO neurons. Together these findings suggest that leptin influences separate subpopulations of neurons that may influence the hypothalamic regulation of feeding, enhancing its ability to inhibit food intake; however, currently it is not known whether leptin signaling in the SFO affects energy balance.

Changes in circulating leptin concentrations clearly influence feeding behavior and the activity of neurons in regulatory centers in the hypothalamus, including the ARC. The actions of leptin on neurons in the ARC is well documented, with depolarizing and hyperpolarizing effects observed on specific neuronal populations (13). Whole animal studies have demonstrated Fos activation in ARC neurons following systemic leptin administration, while the excitability of ARC neurons is, unquestionably, influenced by direct leptin application in hypothalamic slices (12). However, an issue that must be taken into consideration is how leptin gains access to central feeding circuits protected behind the BBB. It has been suggested that leptin directly accesses the ARC through a leaky BBB; how-

ever, anatomical studies have clearly demonstrated that the ARC has an intact BBB as it does not contain type III fenestrated capillaries (45). Although a saturable blood-to-brain transport system for leptin has been demonstrated (2), it is not clear what concentrations of transported substances are delivered to their target normally protected by the BBB.

Perspectives and Significance

The SFO, a sensory CVO, possesses fenestrated capillaries permitting circulating leptin direct access to neurons within this central nervous system structure. The lack of the normal BBB and, therefore, direct access to peripheral signals mean that the SFO is well suited for a role in monitoring circulating signals indicating energy status. In addition, the well-documented projections from the SFO to hypothalamic nuclei with established roles in feeding behavior, including the ARC, lateral hypothalamus, and paraventricular nucleus (29, 42), suggest potential roles for SFO efferents in the regulation of energy balance. The present study identifies the SFO as a possible central nervous system location with direct access to the peripheral circulation at which leptin may act to influence hypothalamic metabolic control centers. Our data show that the signaling form of the leptin receptor is present in the SFO and the responsiveness of SFO neurons to leptin; however, the physiological relevance of these observations remains to be fully explored.

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