Central control of energy balance depends on the ability of proopiomelanocortin (POMC) or agouti-related protein (Agrp) hypothalamic neurons to sense and respond to changes in peripheral energy stores. Leptin and insulin have been implicated as circulating indicators of adiposity, but it is not clear how changes in their levels are perceived or integrated by individual neuronal subtypes. We developed mice in which a fluorescent reporter for PI3K activity is targeted to either Agrp or POMC neurons and used 2-photon microscopy to measure dynamic regulation of PI3K by insulin and leptin in brain slices. We show that leptin and insulin act in parallel to stimulate PI3K in POMC neurons but in opposite ways on Agrp neurons. These results suggest a new view of hypothalamic circuitry, in which the effects of leptin and insulin are integrated by anorexigenic but not by orexigenic neurons.

Introduction
Control of energy homeostasis requires the central nervous system to sense and respond to changes in peripheral energy stores. Among first-order neurons suggested to date are those found in the hypothalamic arcuate nucleus that express either proopiomelanocortin (POMC) or agouti-related protein (Agrp), neuropeptides that promote negative and positive energy balance, respectively. The activity of these neurons is regulated in a reciprocal manner by leptin, an adipocyte-derived hormone that conveys afferent input from the periphery to the brain regarding the status of body energy stored in the form of fat (reviewed in refs. 1, 2, 3).

The weight-reducing action of leptin involves both the inhibition of Agrp neurons (which coexpress the potent orexigen neuropeptide Y) and the stimulation of anorexigen POMC neurons. Conversely, deficient leptin signaling in the hypothalamic arcuate nucleus that express either proopiomelanocortin (POMC) or agouti-related protein (Agrp), neuropeptides that promote negative and positive energy balance, respectively. The activity of these neurons is regulated in a reciprocal manner by leptin, an adipocyte-derived hormone that conveys afferent input from the periphery to the brain regarding the status of body energy stored in the form of fat (reviewed in refs. 1, 2, 3).

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neuropeptides, respectively (Figure 1D). To investigate leptin regulation of PI3K signaling in POMC and Agrp neurons, we developed a quantitative approach for measuring membrane.
perfusion with aCSF. Under these conditions, leptin withdrawal triggered membrane accumulation of the PI3K reporter in 18 of 21 Agrp neurons examined (Figure 3B and Figure 4A). Thus, reciprocal regulation of POMC and Agrp neurons by leptin correlates with reciprocal effects on PI3K activation.

The majority of POMC or Agrp neurons responded to leptin addition or leptin withdrawal, respectively, within 10 minutes (Figure 3D). However, the duration of the response varied, with some neurons exhibiting transient membrane localization for less than 1 minute and others exhibiting sustained membrane localization for more than 10 minutes. Overall, the timing of PI3K activation in response to a change in leptin levels was similar in POMC and Agrp neurons whether analyzed according to the cumulative distribution of earliest response (Figure 3D) or the proportion of neurons exhibiting a response within a particular time window (Figure 3E).

PI3K regulation by insulin, and effects of synaptic inhibitors. As described above, leptin and insulin had similar effects on POMC neurons: addition of either compound caused membrane accumulation of the PI3K reporter protein (Figure 2D and Figure 3). Surprisingly, leptin and insulin had opposite effects on Agrp neurons, with membrane accumulation of the reporter protein inhibited by leptin but stimulated by insulin (Figure 4A). The time course of insulin-induced PI3K activation in Agrp neurons appeared similar to that in POMC neurons, with membrane accumulation evident within a few minutes of hormone addition, then declining gradually over 30–60 minutes (Figure 2D and Figure 4A). Unlike POMC neurons, however, activation of PI3K by insulin in Agrp neurons was the opposite of what would be predicted for a physiologic adiposity signal.

To gain further insight into the mechanisms underlying these effects, we asked whether the PI3K response of POMC and Agrp neurons was altered by inhibitors of synaptic transmission. This was accomplished by incubating brain slices in tetrodotoxin (TTX) or reduced extracellular calcium (which block voltage-gated sodium channels and calcium-dependent synaptic vesicle release, respectively) prior to measuring membrane localization of the PI3K reporter protein in response to changes in leptin or insulin.

Neither TTX nor reduced extracellular calcium affected the response of POMC neurons to leptin addition (Figure 4C), but both treatments markedly reduced the response to leptin withdrawal in Agrp neurons (Figure 4D). Thus, leptin directly activates PI3K in POMC neurons, but the effect of leptin withdrawal in activating PI3K in Agrp neurons requires synaptic transmission and therefore must be mediated indirectly. We also tested whether synaptic transmission was required for the effects of insulin on PI3K activation and found that neither reduced extracellular calcium nor TTX diminished PI3K activation in POMC or Agrp neurons (Figure 4, B, E, and F); in fact, reduced extracellular calcium...
Leptin-induced PI3K activation in POMC neurons is independent of Stat3 function. The PI3K pathway has received less attention than the JAK-STAT pathway as an effector of leptin action, in part because JAK-STAT signaling is a prototype for type I cytokine receptors and in part because genetic manipulations that interfere with leptin-induced Stat3 phosphorylation cause obesity (4–6). However, Stat3 acts primarily as a transcription factor (21, 22), and leptin regulation of arcuate nucleus neurons clearly involves actions that are independent of changes in gene expression, including regulation of electrical activity and modulation of PI3K (7, 8, 11, 15, 23).

Because Stat3 has also been reported as serving as an adapter protein that recruits PI3K to the activated type I interferon receptor (24), we determined whether leptin-induced PI3K activation in POMC neurons requires Stat3. We first generated mice in which both hormones activate PI3K, and leptin has been shown to cause membrane depolarization and an increased firing rate. Although previous studies by Ashford and colleagues (11, 26) identified a subset of hypothalamic neurons in which the effects of leptin or insulin on membrane hyperpolarization could be blocked by PI3K inhibitors, our results do not distinguish whether changes in POMC PI3K are independent of or causally related to changes in POMC electrical activity. Both phenomenon occur rapidly and may contribute to acute responses such as neuropeptide release and rapid changes in feeding behavior.

Discussion

Previous electrophysiologic studies have painted an intricate but complex picture of hypothalamic energy balance circuitry. POMC neurons marked with an EGFP transgene are depolarized in response to both leptin (7) and elevated extracellular glucose (20); however, a subset of unmarked hypothalamic neurons selected for the same type of glucose responsiveness was found to be hyperpolarized by leptin (26). Our findings provide a new perspective with respect to the nature of both the response and the circuitry, in which PI3K may contribute to the activation of both Agrp and POMC neurons, but the direction of its regulation by leptin depends on whether the action is mediated directly or indirectly (Figure 5C). Thus, reciprocal regulation of PI3K by leptin provides a unifying hypothesis to explain how this hormone exerts opposing effects on the activity of these 2 key subsets of neurons.

An important implication of our findings has to do with the notion that insulin and leptin play similar roles as peripheral indicators of energy balance. This idea was initially based on observations that insulin circulates and enters the brain at a level proportional to the body fat stores and has been supported by observations that central administration of insulin inhibits food intake and reduces body weight (reviewed in refs. 1, 14) and that neuron-selective insulin receptor knockout mice develop moderate diet-induced obesity (27).

Our results suggest that parallel effects of leptin and insulin on energy balance could be integrated at the level of POMC neurons, where both hormones activate PI3K, and leptin has been shown to cause membrane depolarization and an increased firing rate. Although previous studies by Ashford and colleagues (11, 26) identified a subset of hypothalamic neurons in which the effects of leptin or insulin on membrane hyperpolarization could be blocked by PI3K inhibitors, our results do not distinguish whether changes in POMC PI3K are independent of or causally related to changes in POMC electrical activity. Both phenomenon occur rapidly and may contribute to acute responses such as neuropeptide release and rapid changes in feeding behavior.
However, our observation that insulin and leptin have opposite effects on PI3K activity in Agrp neurons suggests that the functions of these 2 hormones as energy balance signals do not overlap completely. Indeed, differential effects of leptin and insulin on Agrp expression have been described previously in the setting of streptozotocin-induced diabetes, in which leptin, but not insulin administration, normalizes Agrp expression (28, 29). Furthermore, the effects of insulin on neuronal growth and survival exceed what might be expected for a simple adiposity signal, with widespread expression of insulin receptors in many brain regions (30) and a connection between insulin action and neurodegeneration (31, 32). Finally, leptin and the melanocortin system are specific to vertebrates while the effects of insulin on the brain are conserved across all metazoans, with genetic studies in invertebrates pointing to an ancient role for insulin in energy balance as well as aging and growth (33, 34). From this perspective, leptin may serve as a specialized energy balance signal invented during vertebrate evolution whose effects partially overlap with a more ancient role for insulin.

In addition to the differential effects of leptin on PI3K activity in Agrp and POMC neurons, our results indicate that the circuits underlying these effects also differ, since the inhibitory effect of leptin on PI3K in the majority of Agrp neurons requires synaptic transmission. However, previous neuroanatomical studies by us and by others have demonstrated that a subset of Agrp neurons (or Npy neurons in the arcuate nucleus) expresses the long form of the leptin receptor (35–39). This apparent paradox—an indirect response to leptin despite the presence of leptin receptors on some Agrp neurons—could be explained if Agrp neurons require multiple signals to activate PI3K in response to leptin withdrawal, some that are mediated autonomously via a leptin receptor and others that are mediated via synaptic trans-
mission. Alternatively, there may be functional heterogeneity within Agrp neurons if, for example, the subset of Agrp neurons that express the leptin receptor are distinct from those that activate PI3K in response to leptin withdrawal. If so, this latter group of neurons might receive leptin-mediated inhibitory signals from POMC or other neurons via γ-aminobutyric acid (GABA) (40) or other inhibitory neurotransmitters (Figure 5C).

Heterogeneity of POMC neurons might also help to explain the observation that leptin activation of PI3K in POMC neurons does not require Stat3 phosphorylation. A recent neuroanatomical study reported that 37% of POMC cells were immunopositive for phospho-Stat3 after leptin treatment (41), and in our imaging system, leptin activated PI3K in approximately 60% of the POMC neurons. Thus, leptin receptor engagement on distinct subsets of POMC neurons could activate different downstream effectors. Regardless, these results together with earlier work (7, 42) suggest a view of leptin signaling in which there are 2 types of direct intracellular effectors with different actions and possibly different mechanisms of activation. One type, exemplified by Stat3, is likely to mediate responses that require changes in gene expression, whereas a second type, exemplified by the direct effects of leptin on PI3K activation and electrical activity in POMC neurons, is likely to mediate acute responses such as neuropeptide release and rapid changes in feeding behavior. In contrast to Stat3, whose mechanism of activation by leptin receptor engagement is well characterized (21, 42, 43), the biochemical mechanisms by which leptin activates PI3K and modulates membrane potential in POMC neurons are not yet clear. However, the two may be causally related, since depolarization of POMC neurons by leptin involves a nonselective cation channel (7) and PIP3 has recently been shown to be capable of modulating ion channels (44, 45). In addition, work of Myers and colleagues (6) has shown that animals carrying a modified leptin receptor that cannot activate Stat3, Lepr1138Ser, develop a subset of the same phenotypic abnormalities caused by a null Lepr allele; thus, it will be interesting to examine the effects of leptin on PI3K activation and electrical activity in POMC neurons that carry the Lepr1138Ser mutation.

We chose to focus on POMC and Agrp neurons because of previous work suggesting they serve as primary sensors for circulating adiposity signals. Balthasar et al. (46) recently demonstrated that animals with POMC-specific Lepr deletion develop a mild obesity and metabolic derangement. To the extent that results from different studies can be compared, the effect of the POMC-specific Lepr deletion appears to be less dramatic than that of a complete Pomc, Mc4r, or Lepr deletion (47). These observations suggest that POMC neurons use mediators and/or signaling mechanisms in addition to leptin to control body weight and, furthermore, that the effect of leptin on CNS control of body weight involves neurons in addition to those that express Pomc. In this regard, comparison of POMC-specific Lepr deletion mice with the POMC-specific Stat3 deletion mice could reveal the extent to which leptin signaling in POMC neurons is mediated by Stat3 or other effectors such as PI3K.

The approach introduced here—a combination of transgenic biology and gene therapy that enables dynamic histochemical measurements of neuronal cell signaling recorded by multiphoton microscopy of brain slices—can be applied to additional signaling events and to diverse pathophysiological problems in which the brain measures and responds to changes in environmental parameters including pH, temperature, glucose concentration, and cytokine levels. Advances in image processing and miniaturization should allow events from multiple neurons to be recorded simultaneously from living animals, an advance that could help in the dissection of molecular and cellular events that underlie complex behaviors.

Methods

Recombinering and generation of transgenic mice. The BAC we used, clone 389J9 (Research Genetics Inc.), contained approximately 60 kb of 5’ and approximately 30 kb of 3’ flanking sequence and was modified by homologous recombination in bacteria using the 2-step RecE and RecT-mediated recombination (ET cloning) method of Stewart and colleagues (48), in which a neomycin resistance cassette flanked by FLP recombinase target sites was used for positive selection and then excised by transient expression of FLP recombinase. Cre recombinase coding sequences modified to contain an optimal translational initiation site and nuclear localization signal were derived from a plasmid originally constructed by M. Lewendoski (National Cancer Institute — Frederick Cancer Research and Development Center, Frederick, Maryland, USA), pML78. A 1.1 kb Sall-EcoRI fragment from pML78 that includes approximately 35 nt of 5’ untranslated sequence and approximately 20 nt of 3’ untranslated sequence was fused to a 0.6 kb EcoRI fragment from the mouse protamine 1 gene that contains an intron and polyadenylation signal (49), and this CreLoxP fragment was then placed upstream of an FLP-flanked neomycin resistance cassette. We placed 50 bp Pomc homology arms at both ends of this cassette, electroporated the linear fragment into bacteria carrying BAC clone 389J9, screened neomycin-resistant clones by PCR and restriction digest analysis for correct targeting, and then removed the neomycin cassette with FLP recombinase. The homology arms were chosen so as to insert the CreLoxP cassette within exon 2 precisely upstream of the normal Pomc translational initiation site; the 5’ and 3’ homology arms correspond to residues 102–151 and 152–201, respectively, from the Pomc cDNA (GenBank accession number NM_00889) — the normal Pomc translational initiation site lies at residue 152–154.

For Agrp, we identified a BAC from a C57BL/6j library (Genome Systems Inc.), clone 171n11, that carried approximately 67 kb of 5’ and approximately 37 kb of 3’ flanking sequence. Using the same strategy as for Pomc, we targeted the CreLoxP cassette precisely upstream of the normal Agrp translational initiation site in exon 2; the 5’ and 3’ homology arms correspond to residues 107088027–107087978 and 107087977–107087928 in the mouse genome sequence of chromosome 8 (mm4, Oct 2003, National Center for Biotechnology Information [NCBI] build 32) (Agrp is encoded in a reverse orientation with a translational initiation site at residues 107087977–107087975).

For both Pomc and Agrp BACs, linear fragments were purified for microinjection since the BAC vector backbone, pBelO Bac11, contains a loxP site. For Pomc, the entire insert was released by digestion with restriction enzyme NotI and purified as an approximately 95 kb fragment using a 20–35% sucrose gradient. For Agrp, there are 2 internal Not sites, and we purified a 48.5 kb NotI fragment using a 20–30% sucrose gradient that contains approximately 47 kb of 5’ and approximately 0.7 kb of 3’ flanking sequence. Linear BAC fragments recovered after sucrose gradient purification were buffer-exchanged with microinjection buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 7.4) and concentrated using centrpipe-30 columns before microinjection (at a concentration of 0.6 μg/ml) into 1-cell embryos produced by mating males homozygous for the R26R allele with standard mouse inbred strain FVB/N females. Transgenic founders were identified and confirmed by PCR and Southern blot hybridization. We found that 22 of 26 Tg.PomcCre lines and 4 of 6 Tg.AgrpCre lines expressed Cre recombinase in the arcuate nucleus of the hypothalamus. In addition, approximately 30% of animals carrying the AgrpCre transgene exhibited early embryonic expression of Cre recombinase as determined by widespread X-gal staining of multiple somatic tissues; these animals were not used for further experiments.
X-gal staining and immunohistochemistry. For X-gal staining of brain slices, 500 μm coronal sections were prepared from freshly dissected brains using an adult brain matrix (Kent Scientific), fixed with 4% paraformaldehyde in 100 mM PBS and 2 mM MgCl₂ (pH 7.4) for 1 hour at 4°C, then washed 3 times, 10 minutes each time, with PBS containing 0.1% Triton X-100 at room temperature. Staining was carried out overnight at 37°C in PBS that contained 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM MgCl₂, and 1 mg/ml X-gal.

For colocalization studies, mice carrying the R26R allele and either a PomC or Agrp transgene were first injected with 1 μl of colchicine (20 μg/μl) into the third ventricle, then anesthetized and perfusion-fixed with 4% paraformaldehyde in 100 mM PBS 48 hours later. Brains were removed, placed in fixative overnight, and infiltrated with 30% sucrose in PBS at 4°C, and then 10 μm frozen sections were prepared and mounted on Superfrost/Plus slides (Fisher). Sections were washed with 0.1% Triton X-100 in PBS (3 times, 10 minutes each time), then incubated with X-gal staining buffer (see above) overnight at 37°C. After X-gal staining, sections were washed with 0.25% Triton X-100 in PBS (3 times, 10 minutes each time), incubated in 0.3% hydrogen peroxide and 0.3% normal goat serum in PBS for 5 minutes at room temperature, washed in 0.25% Triton in PBS, then incubated in 10% normal goat serum and 1% BSA in PBS for 1 hour at room temperature. Sections were subsequently immunostained with a rabbit polyclonal α-melanocyte-stimulating hormone (α-MSH) antibody (Immunostar) at a 1:500 dilution in blocking buffer, or a rabbit polyclonal AGRP antibody raised against human AGRP (35) at a 1:1000 dilution for 1 hour at room temperature. Sections were washed with 0.25% Triton X-100 in PBS (3 times, 10 minutes each time), then incubated with biotinylated goat-anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories Inc.) at 1:500 dilution in blocking buffer for 1 hour at room temperature. After washing with 0.25% Triton in PBS (3 times, 10 minutes each time), we detected biotin using a horseradish peroxidase detection kit (Vector Laboratories).

Cell boundaries of immunostained Agrp neurons were difficult to assess due to the high density of cell bodies and neuronal fibers in the medial portion of the arcuate nucleus, which prevented a quantitative estimate for colocalization. However, results from a different study using the same AgrpCre transgenic line indicated that Cre recombine was expressed in the majority of Agrp neurons (A.W. Xu and G.S. Barsh, unpublished data). Immunostained POMC neurons were distributed in a manner such that the boundaries of individual cells were readily apparent; in a sample of multiple sections from Tg.PomcCre mice (594 cells counted), 79.5% of the cells were doubly positive for X-gal staining and MSH immunoreactivity, 18.4% of the cells were MSH-immunoreactive but did not exhibit X-gal staining, and 2% of the cells were X-gal-positive but not MSH-immunoreactive. Because of the nature of the R26R reporter, lacZ expression provides information on the site that is required for normal Stat3 function.

Southern blotting. For Southern blotting, 2 μg of genomic DNA was electrophoresed on 1% agarose gels at a constant voltage of 100 V for 1 hour. DNA was transferred from the gel to a nylon membrane (Hybond-N+) by capillary transfer. Blots were hybridized with a 32P-labeled 1.3-kb XbaI fragment of the lacZ gene (as a probe) overnight at 65°C. After hybridization, blots were washed twice for 5 minutes each time at room temperature, washed once for 5 minutes at 55°C, twice for 5 minutes at 65°C, and once for 30 minutes at 65°C in 0.1×SSC (0.15 M NaCl, 0.015 M sodium citrate) containing 0.1% SDS. Blots were exposed to Hyperfilm-MP (Amersham) and processed as recommended by the manufacturer.

Immunohistochemistry. The majority of Agrp neurons (A.W. Xu and G.S. Barsh, unpublished data).

Animals with POMC-specific Stat3 deletion were generated by intercrossing Tg.PomcCre and Stat3flox/null mice. The 4 possible Stat3flox/null/Stat3flox/null and null (Stat3null) alleles have been described previously. In brief, the loxP sites flank exon 22, which encodes a phosphorylation site that is required for normal Stat3 function.

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Data analysis. Image files generated during laser scanning were converted into a TIFF format and then analyzed with the Canny edge detection algorithm in MatLab (Version 6.5, release 13). For each series of images, a threshold for edge detection was chosen empirically so that there were no edges detected on the cytoplasmic membrane in images taken prior to hormone treatment. Each image was then edited to remove edges surrounding the nuclear membrane and imported back into MatLab to determine the total number of edge pixels.

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