Functional Role of c-Jun-N-Terminal Kinase in Feeding Regulation

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c-Jun-N-terminal kinase (JNK) is a signaling molecule that is activated by proinflammatory signals, endoplasmic reticulum (ER) stress, and other environmental stressors. Although JNK has diverse effects on immunological responses and insulin resistance in peripheral tissues, a functional role for JNK in feeding regulation has not been established. In this study, we show that central inhibition of JNK activity potentiates the stimulatory effects of glucocorticoids on food intake and that this effect is abolished in mice whose agouti-related peptide (AgRP) neurons are degenerated. JNK1-deficient mice feed more upon central administration of glucocorticoids, and glucocorticoid receptor immunoreactivity is enhanced in the AgRP neurons. JNK inhibition in hypothalamic explants stimulates Agrp expression, and JNK1-deficient mice exhibit increased Agrp expression, heightened hyperphagia, and weight gain during refeeding. Our study shows that JNK1 is a novel regulator of feeding by antagonizing glucocorticoid function in AgRP neurons. Paradoxically, JNK1 mutant mice feed less and lose more weight upon central administration of insulin, suggesting that JNK1 antagonizes insulin function in the brain. Thus, JNK may integrate diverse metabolic signals and differentially regulate feeding under distinct physiological conditions. (Endocrinology 151: 0000–0000, 2010)

Obesity and type 2 diabetes are now considered chronic inflammatory conditions that involve regulatory machinery of the immune system. It is generally accepted that conditions associated with obesity such as increased fatty acids, microhypoxia, endoplasmic reticulum (ER) stress, and cytokines activate proinflammatory responses in peripheral insulin target tissues by activating the c-Jun N-terminal kinase (JNK) and inhibitor of nuclear factor κB kinase (IKK)/nuclear factor κB pathways, which in turn down-regulate insulin signaling (1). In contrast, the central role of inflammatory pathways in the regulation of energy homeostasis is not well understood. Previous studies have shown that many proinflammatory cytokines inhibit feeding when administered centrally (2, 3). Injection of lipopolysaccharide (LPS), a bacterial endotoxin that stimulates synthesis and release of multiple cytokines, also causes anorexia by acting in the central nervous system (CNS) (4–7). Furthermore, recent evidence suggests that IL-1β exerts its anorexigenic effect by acting on proopiomelanocortin (Pomc) and agouti-related protein (AgRP) neurons (4, 6, 7), key hypothalamic neurons that promote negative and positive energy balance, respectively (8). In contrast to the anorexigenic effect of proinflammatory molecules, antiinflammatory signals, such as glucocorticoids, are known to stimulate feeding. Corticosterone administration to corticosterone-deficient mice stimulates food intake (9–11). In addition, adrenalectomy reverses hyperphagia in leptin-deficient ob/ob mice (12). In humans, hypercortisolism, as seen in Cushing’s syndrome or by exogenous administration of glucocorticoids to treat inflammatory diseases, causes obesity (13). More recently, several studies found that exposure to high-fat diet activates IKK-β and nuclear factor-κB signaling and ER stress in the brain, causing hypothalamic leptin resistance and increased feeding (14–16). Taken together, the above studies suggest that activation of central inflammatory pathways in response to different physiological conditions could have diverse effects on energy balance. Elucidating
the functional interactions of proinflammatory and anti-inflammatory pathways, and their neuronal targets will help us understand how these pathways are integrated to affect energy homeostasis.

JNK is a serine kinase that is activated by environmental stressors and ER stress and by multiple metabolic stimuli, such as cytokines, glucocorticoids, and free fatty acids (17–19). Many of these signals are dynamically regulated in various nutritional states and have been shown to act on hypothalamic neurons to affect feeding. There are three isoforms of JNK, and among them, JNK1 is ubiquitously expressed. However, mice deficient for JNK1, but not other JNK isoforms, are resistant to diet-induced obesity and insulin resistance (20, 21). Importantly, a recent study by Solinas et al. (22) shows that JNK1 in hematopoietically derived cells contributes to diet-induced inflammation and insulin resistance without affecting obesity, suggesting that JNK1 acts in other tissues to regulate energy homeostasis. Interestingly, in vitro biochemical studies have shown that JNK1 directly phosphorylates glucocorticoid receptor (GR) on serine residues and inhibits its function by affecting protein stability, nuclear translocation, sumoylation, and transcriptional activity (23–26). Thus, JNK, a proinflammatory signaling component, could be involved in feeding regulation by modulating the activities of antiinflammatory glucocorticoid signaling in the brain. In this study, we show that JNK1 exerts a negative role on energy balance by antagonizing glucocorticoid signaling on hypothalamic AgRP neurons. We propose a model to address the distinct roles of JNK1 in feeding regulation under specific physiological conditions.

Materials and Methods

Mice

The generation of Tg.AgRP-Cre; Git(Rosa)26Soer^tm1Sor (R26R-lacZ) was described previously (27), and these mice have been validated by multiple studies (10, 15, 27–31). As described before (27), about 20–30% of Tg.AgRP-Cre/R26R-lacZ mice exhibit early embryonic expression of Cre as determined by widespread X-gal staining in somatic tissues using an ear biopsy procedure, and these mice were excluded from our study. Mice with deletion of mitochondrial transcription factor A (Tfam) gene specifically in the AgRP neurons (AgRP-Tfam mutants) have previously been developed and characterized in detail (10). In these mice, about 85% of AgRP neurons undergo gradual degeneration by 7 months of age. Eight-month-old female AgRP-Tfam mutants and littermate controls were used in this study. To generate AgRP-Tfam mutant and control mice, males homozygous for the floxed Tfam allele and heterozygous for the AgRP-Cre transgene were mated to females that were homozygous for the floxed Tfam allele. All mice were genotyped as previously described (10). Mice that are heterozygous for a null allele of mapk8 (mapk8^+/−) were purchased from The Jackson Laboratory (Bar Harbor, ME). The mice obtained were on a C57BL/6 background and have been maintained on the same background. No phenotypic difference was observed in wild-type (Mapk8^+/+) and heterozygous (Mapk8^+/−) animals in experiments described in the study, so they were pooled together as controls. For all experiments, age- and sex-matched controls and mutants (Mapk8^−/−) were used. Mice expressing humanized renilla green fluorescent protein (hrGFP) under the control of the mouse Npy promoter were purchased from The Jackson Laboratory [B6.FVB-Tg[NPY-hrGFP]1Low/J], and the specificity of GFP expression has been validated (32). All mice were housed in the University of California, San Francisco, mouse barrier facility in a room with 12-h light, 12-h dark cycle (0700–1900 h light). All experiments were carried out under a protocol approved by the University of California, San Francisco, Institutional Animal Care and Use Committee.

Refeeding studies

For feeding studies, mice were singly housed for at least 1 wk before food intake measurements. To measure compensatory refeeding, food was removed right before the onset of the dark cycle, and mice were fasted for 36 h with free access to water, after which time food was returned. Food intake and body weight were measured 2, 4, 6, 24, 48, and 72 h and 1 wk during refeeding.

Hypothalamic explant culture

Hypothalamic slice cultures were performed as previously described with minor modifications (33–35). Adult control mice were killed, and fresh coronal sections (500 μm) were cut using a brain matrix on ice in dissection medium [50% DMEM, 50% Hanks’ balanced salt solution, 25 mm HEPES buffer, 10 mm Tris-HCl (pH 7.4), and 100 μg/ml penicillin-streptomycin]. Each coronal section through the hypothalamus was cut down the midline along the third ventricle to yield two identical halves. Each half was placed in Millicell CM 0.4-μm culture plate inserts and cultured at 37°C in culture medium (50% DMEM, 25% NPY-hrGFP/1Lowl/J], and the specificity of GFP expression has been validated (32). All mice were housed in the University of California, San Francisco, mouse barrier facility in a room with 12-h light, 12-h dark cycle (0700–1900 h light). All experiments were carried out under a protocol approved by the University of California, San Francisco, Institutional Animal Care and Use Committee.

Intracerebroventricular (icv) injection

For implantation of the guide cannula, mice were anesthetized with 100 mg/kg ketamine (Ketaset; Fort Dodge Animal Health, Fort Dodge, Iowa) and 5 mg/kg xylazine (Akorn Inc., Lincolnshire, IL) with 0.5% isoflurane used as needed to maintain a surgical plane of anesthesia. Custom 5.7-mm guide canulas (Plastics One, Wallingford, CT) were implanted using a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA) into the third ventricle (x, 0.0; y, bregma −2.0; z, −5.7). Buprenorphine (Buprenex; Reckitt Benckiser Healthcare Ltd., Slough, UK) 0.1 mg/kg was used immediately after surgery and then every 12 h for 3 d.
as needed. Mice were singly housed and allowed 1 wk to recover before checking placement with drinking response to angiotensin II (Sigma; 100 µg/ml, 1 µl per mouse). Cannula placement was also verified by postmortem histochemical examination. For ivc injection, a custom 5.9-mm injector (Plastics One) was used. Mice were allowed free movement within a limited area while 1 µl was infused at a rate of 10 nl/sec using a micropump (World Precision Instruments, Sarasota, FL). Mice were injected with artificial cerebrospinal fluid (aCSF), consisting of 150 mM NaCl, 3.0 mM KCl, 1.4 mM CaCl2, 0.8 mM MgCl2, 1 mM NaH2PO4 (pH 7.4), once daily for 3 d, and 1 or 5 µl insulin (Humalin R, Hospira, Inc., Morgan Hill, CA) or 250 ng dexamethasone (water soluble; Sigma) on the fourth day. For SP600125 treatment, SP600125 (264 ng/mouse in aCSF with 33% DMSO) and its corresponding vehicle (33% DMSO in aCSF) was injected with or without dexamethasone. All injections were done at 1800–1900 h, and 24-h food intake was measured after injection and compared with average food intake during the corresponding period of vehicle treatment.

**LPS injection, leptin sensitivity test, and ip injection of corticosterone**

For LPS injection, each mouse was injected ip with saline for 3 d, once daily (1000 h) and then injected with 20 or 50 µg/kg LPS (Sigma) on the fourth day, and 24-h food intake was measured after injection and compared with average food intake during saline treatment. For leptin sensitivity measurement, each mouse was injected ip with saline for 3 d, twice daily (0830 and 1830 h) and then injected with leptin (R&D Systems, Inc., Minneapolis, MN) twice at a dose of 1.5, 2, or 3 mg/kg on the fourth day. Food intake was calculated as the average of the 3-d saline treatment and compared with the 24-h leptin treatment period. For ip injection of corticosterone, control and mutant animals were injected ip once daily for 3 d with vehicle (4, 8, or 16% ethanol in saline) and one of three different doses of 2, 4, or 8 mg/kg corticosterone on the fourth day. Daily food intake was measured over a 4-h period (1000–1400 h).

**Hormonal measurements**

Blood was collected from fed, fasted (36 h), and refed mice via mandibular vein puncture. Plasma insulin and leptin were measured with an insulin ELISA kit (Alpco Inc., Salem, NH) and a leptin ELISA kit (Crystal Chem, Downer’s Grove, IL) according to the manufacturer’s instructions. To measure blood corticosterone levels, fed and 36-h-fasted, singly housed mice were acclimatized to the procedure room for several hours before checking placement with drinking response to angiotensin II. Mice were singly housed and allowed 1 wk to recover after checking placement with drinking response to angiotensin II. Mice were acclimatized to the procedure room for several hours before checking placement with drinking response to angiotensin II. For ip injection of corticosterone, control and mutant animals were injected ip once daily for 3 d with vehicle (4, 8, or 16% ethanol in saline) and one of three different doses of 2, 4, or 8 mg/kg corticosterone on the fourth day. Daily food intake was measured over a 4-h period (1000–1400 h).

**mRNA expression analysis using real-time RT-PCR**

Measurements of mRNA levels were carried out by quantitative RT-PCR on RNA extracted from dissected hypothalamic tissue. Total RNA from each hypothalamus was purified using TRIzol reagent (Invitrogen, Carlsbad, CA) and an RNeasy mini kit (Qiagen, Valencia, CA) and quantified by spectrophotometry (NanoDrop, Wilmington, DE). One microgram of total RNA sample was reverse transcribed and then PCR amplified using the 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) and SYBR green to measure relative cDNA levels. β-Actin was used as the internal control to normalize expression. Agrp, Pomc, Npy, lepr, and β-actin primers were the same as previously published (36). Later experiments were performed using commercial TaqMan gene expression assays (Applied Biosystems) for Agrp, Npy, Pomc, Crb, Nr3C, and β-actin according to the manufacturer’s protocol and measured as above. Some assays were performed using both SYBR green and TaqMan, and no difference was detected between the two methods.

**Immunohistochemistry and immunofluorescence**

Mice were perfused with 4% paraformaldehyde. Brains were removed, placed in 4% paraformaldehyde at 4 C overnight, and then transferred to 30% sucrose in PBS for 24 h at 4 C. Then 10-µm frozen sections were prepared and mounted on Superfrost/Plus slides. X-gal staining was carried out as previously described (10, 27, 37). Bright-field images were captured using a Zeiss Axioscope imaging system equipped with an AxioCam color digital camera. For immunofluorescence, sections were washed in PBS and unmasked as necessary using either 10 min 0.3% glycine, 10 min 0.3% SDS, or 30 min boiling 0.1% sodium citrate solution and then stained using methods described previously (10, 37). Polyclonal anti-GR (Santa Cruz Biotechnology, Santa Cruz, CA; 1:500 dilution), NeuroTrace red fluorescent Nissl stain (Molecular Probes, Eugene, OR; 1:100 dilution), monoclonal anti-glial fibrillary acidic protein (Sigma; 1:500 dilution), polyclonal anti-neuropeptide Y (anti-NPY) (Peninsula Laboratories, LLC, San Carlos, CA; 1:500 dilution), and polyclonal anti-p-c-Jun (Santa Cruz Biotechnology; 1:100 dilution) were used. Goat antirabbit Alexa488, goat antirabbit Alexa555, and goat antimouse Alexa488 (Molecular Probes) were used for secondary antibody detection. Sections were mounted using Vectashield with 4',6-diamidino-2-phenylindole (DAPI), Hard Mount (Vector Laboratories, Burlingame, CA). Fluorescence images were captured using a Zeiss Axioscope2 imaging system equipped with an AxioCam black and white digital camera.

**Cell counting**

Images to be counted were captured as above using the same exposure time for each and no enhancement. Images were first examined for position and sections within the range bregma −1.90 to −2.30 were included in counting. Images were then modified using ImageJ (National Institutes of Health, Bethesda, MD) for background subtraction. Sections were then blinded, an intensity threshold was set, and cells within the arcuate nucleus were counted independently by two different people.

**Statistics**

Analysis was performed by two-tailed Student’s t test, and groups being compared are described in the figure legends. Values are presented as mean ± SEM. For feeding studies that involved treating the same animals with vehicle and various doses of experimental reagents, repeated-measures ANOVA was employed using SPSS version 14.0 software. Multiple pairwise comparisons were computed using estimated marginal means with least significant different adjustment.
Results

Inhibition of JNK activity in hypothalamic explants stimulates Agrp and Npy expression

To explore the role of JNK in feeding regulation, we first examined whether inhibition of JNK activity in hypothalamic neurons affects expression of leptin receptor (lepr) long form, Pomc, Npy, or Agrp. Coronal hypothalamic slices were cut along the third ventricle to yield two identical halves, which were then cultured. One half of the slice was treated with an SP600125, a JNK inhibitor at a dose previously shown to be effective (38), and the other half was treated with its corresponding vehicle. RNA was extracted from the explants after 30 h treatment and semiquantitative RT-PCR was carried out to measure gene expression. Gene expression from two halves of the same brain was compared. Although SP600125 treatment did not affect the expression of leptin receptor long form or Pomc (Fig. 1, A and B), it caused a significant increase in Npy and Agrp mRNA expression compared with vehicle treatment (Fig. 1, C and D).

Because JNK is known to phosphorylate GR in vitro, and glucocorticoids have been shown to stimulate Agrp and Npy expression (11, 33, 39), we examined whether JNK would play a role in mediating glucocorticoids’ effect on Agrp expression. Treatment with a GR inhibitor, RU-38486, abolished the ability of SP600125 to stimulate Agrp expression (Fig. 1D). The apparent effect of JNK inhibition on Agrp expression in the absence of exogenous glucocorticoids is likely due to the presence of glucocorticoids in the serum that was used in the culture (40). Taken together, these results indicate that JNK and glucocorticoids have opposing effects on Agrp expression in hypothalamic cells and that the effect of JNK inhibition on Agrp expression is GR dependent.

Central inhibition of JNK activity potentiates glucocorticoids’ orexigenic effects in controls but not in mutant mice with degenerated AgRP neurons

Our above results suggest that JNK inhibits Agrp and Npy expression via antagonizing the effects of glucocorticoids. We next investigated whether JNK would alter the effects of glucocorticoids on food intake. It is well known that Agrp and Npy are coexpressed from the same neurons within the arcuate nucleus of the hypothalamus (41). Thus, we employed a transgenic mouse model previously developed in which 85% of AgRP neurons gradually degenerated due to deletion of mitochondrial transcription factor A (Tfam) gene from the AgRP neurons (10). Due to the progressive nature of AgRP neurodegeneration, AgRP-Tfam mutant mice have normal food intake and body weight on a regular diet (10). AgRP-Tfam mutant mice were generated by crossing a Tfam floxed allele with Tg.AgRPCre mice, in which Cre recombinase was specifically expressed in the AgRP neurons, as previously described (10). The specificity of the Tg.AgRPCre animals has been previously characterized and validated by a number of independent research groups (10, 15, 27–31). Guide cannulas were implanted into the third ventricle in control and AgRP-Tfam mutant mice. SP600125 (264 ng) or dexamethasone (250 ng), a synthetic glucocorticoid, was administered icv into the brain of control and weight-matched AgRP-Tfam mutant mice. This dose of icv dexamethasone has been shown to restore food intake in adrenalectomized leptin-deficient ob/ob mice but has no effect on wild-type mice (42, 43). Although this dose of SP600125 or dexamethasone treatment alone did not affect feeding, combined treatment of SP600125 and dexamethasone significantly stimulated feeding in the control but not in AgRP-Tfam mutant animals (Fig. 2). This result strongly suggests that the effect of JNK on food intake is via modulation of glucocorticoid function in the AgRP neurons.

JNK1-deficient mice feed more in response to central administration of glucocorticoids

We next investigated whether glucocorticoids differentially affect feeding in mice that are deficient in JNK1.
Consistent with results described previously (20), adult JNK1 mutant mice (Mapk8<sup>-/-</sup>) are slightly smaller and feed slightly less. However, feeding is comparable to the controls when normalized to body weight (supplemental Fig. 1, published as supplemental data on The Endocrine Society’s Journals Online web site at http://endo.endojournals.org). Guide cannulas were implanted into the third ventricle in control and JNK1 mutant mice. Vehicle or dexamethasone (2.50 ng/mouse) was ivc injected, and 24-h food intake was measured. We found that dexamethasone injection did not affect food intake in controls but stimulated feeding more than 2-fold in JNK1 mutants (Fig. 3A). Intraperitoneal injection of corticosterone (8 mg/kg) similarly stimulated feeding in the mutants but not in the controls (Fig. 3B). This result further supports our notion that JNK1 acts centrally to inhibit glucocorticoids’ effect on food intake.

**JNK1-deficient mice respond normally to the anorexigenic effects of proinflammatory cytokines or leptin**

We next investigated whether JNK1 was required for the anorexigenic effect of LPS, which stimulates the synthesis and release of multiple cytokines. Control and JNK1 mutant mice were injected ip with saline or LPS (20 or 50 μg/kg), and food intake was measured 24 h after injection. We found that LPS inhibited food intake in a dose-dependent manner, and to the same degree in controls and mutants (Fig. 3C), suggesting that JNK1 signaling is not required for LPS-mediated anorexia.

Because leptin receptor belongs to the cytokine receptor family, we examined whether leptin sensitivity is altered in JNK1 mutant mice (44). Control and mutant animals were injected ip with saline or leptin (1.5, 2, or 3 mg/kg) twice daily, and daily food intake was measured. Leptin inhibited food intake in a dose-dependent manner, and to the same extent in controls and mutants (Fig. 3D), suggesting that leptin sensitivity is normal in the mutants.

**JNK1-deficient mice show enhanced nuclear GR immunoreactivity in the AgRP neurons**

Upon glucocorticoid binding, GR translocates from the cytoplasm into the nucleus, where it acts as a transcription factor to regulate gene expression. Fasting is known to cause elevation of circulating corticosterone in mice, which we have confirmed (Fig. 4D). Consistent with increased glucocorticoid levels, we found that fasting in-
duced a 3-fold increase in GR immunoreactivity in the medial-basal hypothalamus of the control animals compared with the fed state (Fig. 4, A and B). The GR immunoreactivity was nuclear, evident by their colocalization with DAPI, which marks nuclei. Furthermore, a nearly 2-fold increase of the GR-positive neurons was detected in the mutants compared with the controls (Fig. 4, A and B). No difference in GR gene (NR3C1) mRNA expression was detected (Fig. 4C), suggesting that increased nuclear GR signal is due to increased nuclear translocation of GR or its increase is confined to only a small subset of cells. In addition, no significant difference in circulating corticosterone levels or hypothalamic CRH (Crh) gene expression was detected in the mutants (Fig. 4, D and E). Together these results suggest that glucocorticoid production is normal in mutant animals.

To verify the identity of these GR-positive neurons, we used the Tg.AgRPcre transgenic mice as described above and crossed them to a Rosa26 Cre reporter line that expresses the LacZ gene in a Cre-dependent manner. Thus, AgRP neurons could be identified by their expression of β-galactosidase, the LacZ gene product (Fig. 5). We found that about half of the AgRP neurons were positive for GR (409 of 853 AgRP neurons); likewise, a significant number of GR-positive neurons were AgRP neurons (409 of 919). Taken together, these data suggest that glucocorticoid function is enhanced in AgRP neurons of the mutant animals, and it is not due to increased circulating ligand
levels. This result is consistent with our notion that JNK1 negatively regulates GR function in the AgRP neurons.

To determine whether development of the hypothalamic feeding center was altered in JNK1 mutant mice, we examined the projection patterns of AgRP neurons in the control and mutant hypothalamus. AgRP is known to co-express with Npy in neurons within the arcuate nucleus of the hypothalamus, and neuronal projections from these neurons were revealed by immunofluorescent analysis using an NPY antibody. No gross difference in the projection patterns was detected (supplemental Fig. 2). We also showed that control and JNK1 mutant mice had a similar number of Nissl-positive cells (382 ± 14 per section in controls vs. 375 ± 10 in mutants) and glial fibrillary acidic protein-positive cells (97 ± 14 per section in controls, 106 ± 8 in mutants) within the arcuate nucleus, suggesting that the JNK1 mutants do not have gross alteration in number of neuronal and glial cell populations within the hypothalamic feeding center (supplemental Fig. 3).

**JNK1 mutant mice have increased Agrp and Npy expression, heightened hyperphagia, and weight gain during refeeding**

We have previously shown that inhibition of JNK activity in hypothalamic explants led to a significant increase in Agrp and Npy expression (Fig. 1). We thus examined whether Agrp and Npy expression was also altered in JNK1 deficiency in vivo. mRNA expression of Agrp, Npy, and Pome from hypothalamic tissues was examined by semiquantitative real-time RT-PCR. We found that Agrp and Npy expression was significantly elevated in the mutants compared with the controls but that Pome expression was similar (Fig. 6, A–C).

Because JNK1 mutant mice are hypersensitive to hypothalamic administration of glucocorticoids, we wished to determine whether mutant animals would show altered feeding under physiological conditions in which glucocorticoid levels are high. Fasting is one such condition where glucocorticoid levels are significantly elevated and adrenalectomy blunts the refeeding response (45). To this end, mice were fasted for 36 h and then allowed free access to food. Weight loss was similar in both genotypes during the fast (Fig. 6G), suggesting that energy expenditure was similar. Food intake during refeeding was measured and compared withprefasted food intake of the same mouse, so each mouse served as its own control. As expected, a hyperphagic response was observed in both genotypes during refeeding; however, mutants are significantly more than the controls during refeeding, and this increased feeding persisted for 24 h (Fig. 6, D–F). Food intake in the mutants gradually reduced thereafter and returned to their prefasted levels within 48 h (Fig. 6F). Consistent with the increase in food intake, mutant mice regained their weight significantly faster than the controls during refeeding, and this increased feeding persisted for almost 2 d refeeding (Fig. 6G), suggesting that energy expenditure was similar. Blood glucose levels also changed appropriately in controls and mutants after fasting and under different feeding conditions (Fig. 6, H and I). Examination of insulin and leptin levels revealed that they changed appropriately in controls and mutants after fasting and under different feeding conditions (Fig. 6, H and I).

**JNK activity is reduced during fasting**

We next investigated whether JNK activity in the AgRP neurons could be modulated by fasting by examining phosphorylation of serine-73 at the N terminus of the c-Jun protein, a site that is predominantly phosphorylated by JNK (46). Because Agrp and Npy are coexpressed by

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**FIG. 5.** Colocalization of GR immunoreactivity with AgRP neurons. A, Hypothalamic sections from 36-h fasted control mice containing Tg.AgRP-Cre and the Cre-reporter R26R-LacZ were stained with X-gal to identify AgRP neurons, as marked by expression of β-gal (β-gal, blue staining in cell bodies with characteristic perinuclear dots). B, GR immunoreactivity in the same section as shown in A. C–E, The zoomed image of the boxed area in A was taken with the fluorescent microscope's bright-field lamp to ensure colocalization and pseudo-colored to red (C). Fluorescent GR signal from the same field (D) and merged image (E) are shown. F, DAPI stain reveals nuclei of all cells in the hypothalamus, and neuronal projections from these neurons were revealed by immunofluorescent analysis using an NPY antibody. No gross difference in the projection patterns was detected (supplemental Fig. 2). We also showed that control and JNK1 mutant mice had a similar number of Nissl-positive cells (382 ± 14 per section in controls vs. 375 ± 10 in mutants) and glial fibrillary acidic protein-positive cells (97 ± 14 per section in controls, 106 ± 8 in mutants) within the arcuate nucleus, suggesting that the JNK1 mutants do not have gross alteration in number of neuronal and glial cell populations within the hypothalamic feeding center (supplemental Fig. 3).
the same neurons within the arcuate nucleus, we used transgenic mice in which hrGFP was specifically expressed in the AgRP/NPY neurons such that these neurons could be readily identified by GFP expression (32). Immunofluorescence analysis was performed to visualize phosphorylation of c-Jun in AgRP/NPY neurons from mice that were either fed or fasted for 25 h. The percentage of AgRP/NPY neurons that were positive for phospho-Ser73-c-Jun was moderately decreased in fasting compared with the fed condition, and this decrease was statistically significant (Fig. 7). The reduction of JNK activity during fasting is consistent with our notion that reduced JNK activity during fasting removes a negative tone on the GR, which in turn stimulates Agrp expression and sets the stage for hyperphagic refeeding.

FIG. 6. JNK1-deficient mice exhibit increased Agrp expression, heightened hyperphagia, and weight gain during refeeding. A–C, mRNA expression was analyzed by semiquantitative RT-PCR from hypothalamic tissues of 16- to 19-wk-old male mice that were fed or fasted for 36 h. β-Actin was used as an internal control. Expression was normalized to control fed value. Control n = 6; mutant n = 5. D–F, Mice were fasted for 36 h and then allowed free access to food. Food intake was measured during the refeeding period (D) and compared with the prefasted 24-h food intake of the same mouse (E and F). G, Body weight change of control and JNK1 mutant mice after fasting and during the refeeding period. Control n = 12; mutant n = 11. *, P < 0.05; **, P < 0.01 between controls and mutants as determined by Student’s t test (A–G). H and I, Plasma insulin and leptin levels were measured by ELISA under fed, 36 h fasting, and 6 h refeed conditions. Insulin n = 15 each for control and mutant under fed and refeed conditions; control n = 6, and mutant n = 9 under fasting condition. Leptin control n = 13–14, and mutant n = 15 under fed and refeed conditions; control n = 7, and mutant n = 8 under fasting condition.

FIG. 7. Phosphorylation of serine-73 at the c-Jun N-terminal domain is reduced in the AgRP/NPY neurons during fasting. A and B, Mice expressing NPY-GFP were either fed (A) or fasted for 25 h (B). Immunofluorescence analysis was performed on hypothalamic sections to detect phosphorylation of serine-73 (red, nuclear signal) at the c-Jun N terminus, a site that is predominantly phosphorylated by JNK (46). Direct GFP fluorescence (green, in both cytoplasm and nucleus) marks the AgRP/NPY neurons. C, Quantification of colocalized cells shows reduced phospho-c-Jun (pc-Jun) at serine-73 in the AgRP/NPY neurons in the fasted state. n = 10 sections from three fed mice, and n = 9 sections from three fasted mice. *, P < 0.05 between controls and mutants as determined by Student’s t test. 3V, Third ventricle.
JNK1-deficient mice are hypersensitive to the anorexigenic effects of insulin

The above results suggest that JNK1 acts as a negative regulator of energy balance. However, this presents a paradox because JNK1-deficient mice are resistant to diet-induced obesity (20), suggesting that JNK1 acts as a positive regulator of energy balance. Because JNK1 is known to inhibit insulin signaling in peripheral tissues, we investigated whether JNK1 would inhibit insulin function in the brain. To this end, insulin was administered into the brain of control and JNK1 mutant mice via third ventricle cannula. A high dose of insulin (5 mU/mouse) caused a significant decrease in food intake and body weight in both controls and mutants, consistent with the anorexigenic effects of insulin. However, a low dose of insulin (1 mU/mouse) had no effect on food intake and body weight in the controls but caused a significant decrease in food intake and body weight in the mutants (Fig. 8). This result indicates that JNK1 acts in the brain to antagonize the effects of insulin on food intake, suggesting that central JNK1 deficiency could enhance the anorexigenic effects of insulin and prevent diet-induced obesity. This notion is consistent with the findings by De Souza and colleagues (47) that central inhibition of JNK activity enhances insulin signaling and decreases food intake in diet-induced obese rats.

Discussion

In this study, we investigated the function of JNK1 in feeding regulation. We provide evidence that JNK1, a proinflammatory signaling molecule, plays a novel role in feeding regulation by acting on hypothalamic neurons. JNK1 exerts an inhibitory effect on glucocorticoid signaling in the AgRP neurons, thereby reducing Agrp expression and feeding. The lack of response of AgRP-Tfam mutant mice to central JNK inhibition suggests that AgRP neurons are essential mediators of JNK1 function. It should be noted that both AgRP and NPY functions in the arcuate nucleus are disrupted in the AgRP-Tfam mutant mice due to the coexpression of these two genes. So it is currently unclear whether JNK1 differentially regulates AgRP or Npy within the same neurons, although glucocorticoids have been shown to affect the expression of both genes (33, 39). Both AgRP and NPY are potent orexigenic neuropeptides, and overexpression of AgRP leads to profound hyperphagia and obesity (48). Situated in the mediobasal hypothalamus and adjacent to the median eminence, a circumventricular organ, AgRP neurons are in constant communication with blood-borne metabolic and hormonal signals. We have previously shown that AgRP neurons mediate both leptin and insulin signaling (27, 49). In addition, AgRP neurons have been shown to be direct targets of IL-1β (4). Thus, AgRP neurons are key neuronal targets to sense and integrate diverse metabolic, proinflammatory, and antiinflammatory signals. Our study shows that JNK, a canonical proinflammatory signaling molecule, functionally interacts with an antiinflammatory pathway in these neurons to regulate food intake.

The heightened hyperphagia and excess weight gain during refeeding observed in JNK1 deficiency is unique. The ability to regulate feeding in response to environmental and physiological challenges such as starvation is essential for survival. Compensatory refeeding is robustly regulated to ensure that shrunken fat stores can be quickly replenished. To date, a few mouse mutations have been identified with attenuated refeeding (10, 37, 50–52), whereas other models show normal refeeding despite being lean (53) or obese (54). The JNK1 mutant mouse, to our knowledge, represents a unique genetic model with heightened refeeding, indicating that JNK1 plays a unique role in regulating energy balance in times of energy deficit. The fasting-induced reduction of JNK-mediated phosphorylation in the AgRP/NPY neurons lends support to our notion that reduced JNK activity during fasting removes a negative tone on the GR, which in turn stimulates Agrp expression and set the stage for hyperphagic refeeding. Conversely, JNK activity has been shown to increase in the hypothalamus of diet-induced obese rodents (47). Thus, it is possible that hypothalamic JNK is regulated by hormones that reflect the abundance of the body’s energy stores, and future studies will be needed to identify these upstream regulators.
The effect of glucocorticoids on AgRP neuronal function is most evident during refeeding. Upon food deprivation, glucocorticoid levels are dramatically elevated, which is accompanied by a marked increase in Agrp expression and a hyperphagic response upon refeeding. Consequently, adrenalectomy or acute ablation of AgRP neurons leads to reduced refeeding (45, 52). The stimulatory role of glucocorticoids on Agrp expression is well established. It has been shown that adrenalectomy or genetic corticosterone deficiency blunts Agrp expression, and corticosterone replacement rescues Agrp expression and stimulates food intake (9–11, 39). It remains to be determined whether glucocorticoids regulate Agrp expression directly by the transcriptional activity of GR. A conserved glucocorticoid-responsive element is present immediately upstream of the AgRP regulatory region as revealed by sequence analysis using the UCSC Genome Browser version 187 TFBS Conserved feature, but the functional importance of this conserved glucocorticoid-responsive element is unknown. Alternatively, a recent study shows that glucocorticoids up-regulate Agrp and Npy gene expression in the arcuate nucleus through activation of the AMP-activated protein kinase signaling (33).

Our results suggest that JNK1 acts as a negative regulator of energy balance because JNK1 deficiency results in hyperphagia and excess weight gain upon refeeding. However, this presents a paradox because JNK1-deficient mice are also shown to be resistant to diet-induced obesity and insulin resistance (20), suggesting that JNK1 acts as a positive regulator of energy balance. A recent study by Solinas et al. (22) shows that JNK1 in hematopoietically derived cells contributes to diet-induced inflammation and insulin resistance without affecting obesity, suggesting that JNK1 acts in other tissues to regulate energy homeostasis. Our current study indicates that JNK1 acts in the brain to antagonize the effects of insulin on food intake, suggesting that central JNK1 deficiency could enhance anorexigenic effects by insulin and prevent diet-induced obesity. This notion is consistent with the findings by De Souza and colleagues (47) that central inhibition of JNK activity enhances insulin signaling and decreases food intake in diet-induced obese rats.

To reconcile the opposing effects of JNK1 deficiency on feeding, we propose a model where JNK1 regulates food intake by sensing and integrating glucocorticoid and insulin signaling in hypothalamic neurons. This dichotomy stems from the differential roles of JNK on CNS insulin and glucocorticoid signaling under different nutritional status. Under conditions of positive energy balance such as normal growth or high-fat feeding, JNK acts to repress CNS insulin signaling. It has been shown that insulin directly and potently inhibits AgRP neuronal activity (30). Thus, JNK deficiency would result in enhanced insulin signaling in the hypothalamus, leading to hyperpolarization of AgRP neurons and consequently a lean phenotype. Under conditions of negative energy balance such as fasting, insulin levels decline precipitously, removing an inhibitory tone on the AgRP neurons. In the meantime, glucocorticoid levels increase, whereas JNK activities decrease. These combined effects lead to activation of GR function, increased Agrp expression, and heightened refeeding. Taken together, these results suggest that JNK1 can act as a negative or a positive regulator of feeding under different physiological conditions by integrating the relative contributions of glucocorticoids, insulin, and other metabolic signals in the brain.

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**References**

10. Xu AW, Kaelin CB, Morton GJ, Ogemoto K, Stanhope K, Graham...


Mol Pharmacol 74:1610–1619.


Collective and individual functions of leptin receptor modulated glucocorticoid receptor transcriptional activation by the c-Jun N-terminal kinase. Proc Natl Acad Sci USA 95:2050–2055.


2003 Role of glucocorticoids in mediating effects of fasting and diabetes on hypothalamic gene expression. BMC Physiol 3:5


52. Luquet S, Phillips CT, Palmiter RD 2007 NPY/AgRP neurons are not essential for feeding responses to glucoprivation. Peptides 28: 214–225
