Synergetic Interaction between Leptin and Cholecystokinin in the Rat Nodose Ganglia Is Mediated by PI3K and STAT3 Signaling Pathways

IMPLICATIONS FOR LEPTIN AS A REGULATOR OF SHORT TERM SATIETY

Andrea Heldsinger, Gintautas Grabauskas, Il Song, and Chung Owyang

From the Division of Gastroenterology, Department of Internal Medicine, University of Michigan, Ann Arbor, Michigan 48109

Research has shown that the synergistic interaction between vagal cholecystokinin-A receptors (CCKARs) and leptin receptors (LRbs) mediates short term satiety. We hypothesize that this synergistic interaction is mediated by cross-talk between signaling cascades used by CCKARs and LRbs, which, in turn, activates closure of K⁺ channels, leading to membrane depolarization and neuronal firing. Whole cell patch clamp recordings were performed on isolated rat nodose ganglia neurons. Western immunoblots elucidated the intracellular signaling pathways that modulate leptin/CCK synergism. In addition, STAT3, PI3K, Src, and MAPK genes were silenced by lentiviral infection. Patch clamp studies showed that a combination of leptin and CCK-8 caused a significant increase in membrane input resistance compared with leptin or CCK-8 alone. Silencing the STAT3 gene abolished the synergistic action of leptin/CCK-8 on neuronal firing. Leptin/CCK-8 synergistically stimulated a 7.7-fold increase in phosphorylated STAT3 (pSTAT3), which was inhibited by AG490, C3 transferase, PP2, LY294002, and wortmannin, but not PD98059. Silencing the Src and PI3K genes resulted in a loss of leptin/CCK-stimulated pSTAT3. We conclude that the synergistic interaction between vagal CCKARs and LRbs is mediated by the phosphorylation of STAT3, which, in turn, activates closure of K⁺ channels, leading to membrane depolarization and neuronal firing. This involves the interaction between CCK/Src/PI3K cascades and leptin/JAK2/PI3K/STAT3 signaling pathways. Malfunctioning of these signaling molecules may result in eating disorders.

Leptin, the product of the ob gene, is secreted primarily from white adipocyte tissue; its level in the circulation correlates with the degree of adiposity (1, 2). Circulating leptin crosses the blood-brain barrier via a receptor-mediated transport system (3, 4) and acts on the long form of the leptin receptor (LRb)⁵ in the medial hypothalamus to regulate feeding behavior and energy balance (5). Leptin is secreted from several other sites, including the gastric mucosa, brown adipocyte tissue, placenta, mammary gland, ovarian follicles, and brain (5, 6). Leptin mRNA and leptin protein have also been detected in human stomach mucosa (7) and rat gastric fundus (8). Leptin levels in the stomach are altered by nutritional state and by cholecystokinin (CCK) administration. CCK is not, however, a stimulus for leptin release from isolated adipocytes (8). Leptin is the key signaling molecule responsible for long term satiety and energy balance; mutations that cause defective leptin secretion or abnormal leptin receptor signaling result in obesity in ob/ob mice (9, 10) and in humans (11). The leptin receptor belongs to the IL-6 receptor family of class 1 cytokine receptors and mediates the biological effects of leptin via the Janus kinase 2-signal transducer and activator of transcription 3 (JAK2/STAT3) pathway (12–14). Several splice variants of the leptin receptor exist; however, the LRb isoform mediates the leptin effect on satiety (4).

CCK is an endogenous peptide found in the gastrointestinal tract and the brain. It is released into the circulation after a meal and acts on neurons both centrally and peripherally (15). The satiety action of CCK appears to be mediated by low affinity CCK-A receptors (CCKARs) on vagal afferent neurons (16). Systemic administration of CCK inhibits food intake in several species, including rats and humans (17), giving credence to the hypothesis that peripheral CCK acts as a satiety signal. CCK cannot penetrate the blood-brain barrier; therefore, systemically administered CCK likely acts at a peripheral site to inhibit feeding (18). In contrast to leptin, the effect of CCK on food intake occurs within 15 min after intraperitoneal administration of CCK-8, suggesting that CCK may act as a meal-related short term satiety signal (19, 20).

Both CCKARs and LRbs are widely distributed in nodose ganglia (NG) and the vagus nerve (21, 22). There is evidence that a synergistic interaction between leptin and CCK leads to the reduction of short term food intake (23–25). In fact, the satiety action of CCK appears to depend on leptin signaling (26). Currently, the intracellular signaling mechanisms responsible for the synergistic interaction between CCK and leptin are unknown. The low affinity CCKAR is a G protein-coupled receptor that signals via multiple signal transduction pathways, including the extracellular calcium-dependent Src kinase, RhoA, phosphoinositide 3-kinase (PI3K), and mitogen-activated protein kinase (MAPK) pathways (27, 28). In addition to

⁵ This work was supported, in whole or in part, by National Institutes of Health Grants R01 DK 48419 and P30 DK 39433 from NIDDK.
¹ To whom correspondence should be addressed: 3912L Taubman Center, University of Michigan Health System, 1150 E. Medical Center Dr., SPC 5362, Ann Arbor, MI 48109-5362. Tel.: 734-936-4785; Fax: 734-936-7392; E-mail: cowyang@med.umich.edu.
² The abbreviations used are: LRb, leptin receptor b; CCK, cholecystokinin; CCKAR, cholecystokinin-A receptor; EGR1, early growth response factor-1; NG, nodose ganglia.
Leptin and Cholecystokinin Synergistic Interaction

the JAK2/STAT3 pathway, leptin regulates other key signaling pathways, such as the extracellular signal-regulated kinase-1/2 (ERK1/2) signaling pathway via the MAPK cascade, and the PI3K pathway, which is mediated by the insulin receptor substrate (13). Because both PI3K and STAT3 possess Src homology domains, it is conceivable that CCK enhances the phosphorylation of STAT3, which may be the pivotal kinase mediating the synergistic interaction between leptin and CCK, leading to short term satiety. The purpose of this study was to investigate the downstream signaling pathways involved in the synergistic interaction between leptin and CCK in the NG and to evaluate the effect of this synergism on neuronal firing. We hypothesized that this synergistic interaction is mediated by cross-talk between the signaling cascades used by CCKARs and LRbs and that phosphorylation of STAT3 activates closure of K+ channels, leading to membrane depolarization and neuronal firing. We showed that leptin and CCK cause a synergistic increase in PI3K phosphorylation and activation of STAT3. Silencing the STAT3 gene resulted in a loss of neuronal firing in rat NG, providing evidence of a direct link between STAT3 and neuronal firing.

**EXPERIMENTAL PROCEDURES**

**Materials**—CCK-8, rat leptin, and C3 transferase were purchased from Sigma-Aldrich. Antibodies were purchased from Cell Signaling, and Santa Cruz Biotechnologies. PP2, LY294002, wortmannin, AG490, and PD98059 were obtained from Tocris, Ellisville, MO. Small interfering RNAs (siRNAs) were purchased from Santa Cruz Biotechnologies. All acute isolation and tissue culture reagents were purchased from Invitrogen. Western immunoblotting reagents were purchased from Bio-Rad, GE Healthcare, and Thermo Scientific.

**Acute Isolation and Culture of Rat NG Cells**—Adult male Sprague-Dawley rats (200–250 g) were obtained from Charles River Laboratories. The experiments were performed in accordance with National Institutes of Health guidelines and approved by the University of Michigan Committee on Use and Care of Animals. The rats were killed with CO2 asphyxiation, and the NG were dissected and immersed in Hank’s balanced salt solution with Pen-Strep and 0.1% bovine serum albumin (BSA). The NG were minced and digested for 60 min at 37 °C in 4 ml of oxygenated physiological salt solution (pH 7.4) containing 0.1% albumin and 137 mM NaCl, 4.7 mM KCl, 0.56 mM MgCl2, 1.28 mM CaCl2, 1 mM NaHPO4, 10 mM HEPES, 5.5 mM glucose, 0.1 mM nonessential amino acid, and 2 mM glutamate. The NG cells were centrifuged at 900 rpm for 5 min. This step was repeated twice, and the final pellet was resuspended in 4 ml of physiological salt solution (pH 7.4). The cells were incubated with inhibitors for 30 min before a 10-min stimulation with leptin and CCK-8. The reaction was stopped by the addition of 1 ml of chilled buffer (8 mM HEPES, 1 mM sodium orthovanadate, 0.5 mM Na2HPO4, 109.5 mM NaCl, 4.7 mM KCl, 1.3 mM MgCl2). The cells were immediately centrifuged at 10,000 rpm for 15 s at 4 °C. The media were aspirated, and the cells were washed twice with 1 ml of ice-cold phosphate-buffered saline (PBS). After the final wash, lysis buffer (30 μl) with protease inhibitor (Roche Applied Science) was added to the culture dish, and the cells were incubated for 15 min at 4 °C. The lysate was centrifuged at 10,000 × g for 5 min. 10 μl of the supernatant was analyzed for protein estimation using a Bio-Rad protein assay kit, and the remainder was analyzed by Western immunoblotting.

**Electrophysiological Recordings**—Whole cell patch clamp recordings were performed on cultured NG neurons within 36 h of plating. A single culture dish was transferred to the stage of an inverted microscope (Nikon, Diaphot 200). Whole cell patch clamp recordings were recorded at 30 °C ± 0.5 °C. The recordings were obtained using patch pipettes with an access resistance of 3–5 meqohms and an internal solution consisting of 140 mM potassium gluconate, 10 mM HEPES, 10 mM EGTA, 1 mM MgCl2, 1 mM CaCl2, 1 mM ATP, and 0.5 mM GTP; adjusted to pH 7.3 with KOH. The neurons were bathed in an external solution consisting of 140 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 10 mM HEPES, 5 mM glucose, at 300 mOsmost and pH 7.3. Whole cell currents were measured using a patch clamp amplifier (Axopatch 200B; Molecular Devices, Sunnyvale, CA), digitized (DIGIDATA 1322A; Molecular Devices), and recorded with a PC using pCLAMP9 software (Molecular Devices).

**Lipofectamine Transfection with Silencing RNA in Primary Cultured NG**—siRNAs for PI3K p85α (SC-156021), Src-1 (SC-36556), ERK1 (SC-156030), and ERK2 (SC-156031) and a siRNA-A unrelated to these genes to be used as the control (SC-37007) were diluted in Opti-MEM. The final concentration of siRNA added to the cells was 15 nM. NG neurons at 50–60% confluence were transfected with control siRNA-A, PI3K siRNA, ERK1 siRNA, ERK2 siRNA, or SRC-1 siRNA in Opti-MEM and Lipofectamine 2000 for 4–6 h. The media were supplemented to a final volume of 2 ml of low glucose DMEM and F12 medium in equal volumes, supplemented with 10% FBS and 1-glutamine, without antibiotics. The neurons were incubated for 72 h with siRNA. The media were removed and replaced with low glucose serum-free DMEM and F12 medium in equal volumes, supplemented with 10% FBS and 1-glutamine, without antibiotics. The neurons were incubated for 10 min with or without CCK-8 (1 nM) + leptin (1 nM). The media were aspirated, and the neurons were washed twice with 1 ml of ice-cold PBS. After the second wash, lysis buffer (30 μl) with protease inhibitor was added.
to the culture dish, and the neurons were incubated for 15 min at 4 °C. The lysate was centrifuged at 10,000 × g for 5 min. 10 μl of the supernatant was analyzed for protein estimation using a Bio-Rad protein assay kit, and the remainder was analyzed by Western immunoblotting.

**Lentivirus-based Gene Silencing in Primary Culture NG by RNA Interference—STAT3 short hairpin RNA (shRNA) was designed and constructed in lentiviral shRNA vectors to silence the expression of rat STAT3 (NM_012747). STAT3 shRNA sequence was designed with the base shRNA sequence flanked with blunt T/A bp on the 5' end and an XhoI sticky end on the 3' end (sense strand, 5'-Phos-TGGAGGAGGCATTCGGAA-GATATCAAGAGATACCTTCCGAATGCCTCCTCTT-TTTTC-3'; antisense strand, 5'-Phos-ACCTCCTCCGTAAAG-CCTTTCTCATAGTTCTATGAAAGGCCTACGGAGGA-GGAAAAAGAGCT-3').** (Integrated DNA Technologies, Coralville, IA). Both sense and antisense strands were generated by oligonucleotide synthesis with 5′ phosphate and PAGE purification. The shRNAs were subcloned into the Hpfl and Xhol cloning site of the pLentilox3.7 proviral plasmid, containing an upstream U6 promoter and a cytomegalovirus-green fluorescent protein (CMV-GFP) expression cassette. The pLL3.7-STAT3 shRNA plasmid and packaging plasmids (pMLDg/pRRE, pRSV-Rev, pCI-VSVG) were cotransfected into 293T cells by standard calcium phosphate precipitation.

The viral supernatant was aspirated, and the cells were incubated for 15 min at 4 °C. The lysate was centrifuged at 10,000 rpm. The viral pellet was resuspended in DMEM at 10^6 transduction units/ml control. The primary culture NG neurons were infected with 20–100 neurons in 2 ml of DMEM/F12 medium with 10% FBS at 37 °C. For the immunoblot studies, the data were normalized for actin in each experiment and expressed as the fold increase above the unstimulated control. Results from three to eight experiments were used to calculate the means ± S.E. To determine the effects of inhibitors, the data were analyzed using one-way ANOVA, followed by a Dunnett's t test. Statistical significance was set at p < 0.05.

**RESULTS**

**Leptin and CCK-8 Synergistic Depolarization of Cultured Rat NG**—Continuous membrane potential recordings in response to extracellular application of 1 nm leptin or 1 nm CCK-8 in combination were obtained in a patch clamp study in cultured NG. Leptin (1 nm) alone did not produce an effect on neuronal input resistance or resting membrane potential. Application of CCK-8 (1 nm) depolarized the NG neuron at −4 mV but did not reach the threshold for action potential. Simultaneous application of leptin (1 nm) and CCK-8 (1 nm) depolarized the membrane potential which exceeded the threshold for action potential. This was associated with an increase in membrane input resistance. Current-voltage relationship analysis showing that the current reversed at −100 mV for each peptide, alone or in combination, which is close to the K⁺ equilibrium potential (−105 mV) for neuronal firing. The data are representative of three independent experiments.

Leptin and Cholecystokinin Synergistic Interaction

**FIGURE 1.** Leptin and CCK-8 stimulate depolarization of cultured rat NG. **A**, representative continuous membrane potential recordings in response to extracellular application of 1 nm leptin or 1 nm CCK-8 or a combination of 1 nm leptin and 1 nm CCK-8 in a patch clamp study in cultured NG. Leptin (1 nm) alone did not produce an effect on neuronal input resistance or resting membrane potential. Application of CCK-8 (1 nm) depolarized the NG neuron at −4 mV but did not reach the threshold for action potential. Simultaneous application of leptin (1 nm) and CCK-8 (1 nm) depolarized the membrane potential which exceeded the threshold for action potential. This was associated with an increase in membrane input resistance. **B**, current-voltage relationship analysis showing that the current reversed at −100 mV for each peptide, alone or in combination, which is close to the K⁺ equilibrium potential (−105 mV) for neuronal firing. The data are representative of three independent experiments.
synergistic interaction occurred between CCK-8 and leptin, resulting in enhanced depolarization of the NG neurons, and this depolarization was mediated by the K⁺ channels.

**Leptin and CCK-8 Synergistic Stimulation of Phosphorylated STAT3 and PI3K in Rat NG**—A dose-response curve for leptin and CCK-8 (0.1–100 nM) stimulation of phosphorylated STAT3 (pSTAT3) was established. A representative pSTAT3 immunoblot is shown in Fig. 2A. Leptin and CCK-8 each caused dose-dependent increases in pSTAT3, which peaked at 1 nM (Fig. 2B). To determine the synergistic stimulation of pSTAT3 by leptin and CCK-8, acutely isolated rat NG were stimulated for 10 min with subthreshold concentration of 0.01 nM leptin or 0.01 nM CCK-8 or a combination of 0.01 nM leptin and 0.01 nM CCK-8. A representative pSTAT3 immunoblot (Fig. 3A) shows the synergistic increase in pSTAT3 in response to a combination of 0.01 nM leptin and 0.01 nM CCK-8 by 3.6 ± 0.26-fold, whereas leptin or CCK-8 alone failed to stimulate pSTAT3. The data represent means ± S.E. (error bars) from three independent experiments. *, p < 0.05 compared with the response to control, CCK-8, or leptin alone.

**Figure 2.** Leptin and CCK-8 stimulate phosphorylation of STAT3 in a dose-dependent manner. A, representative immunoblot of a leptin and CCK-8 dose response in acutely isolated rat NG stimulated for 10 min with leptin or CCK-8. B, combination of 0.01 nM leptin and 0.01 nM CCK-8 caused peak increases in pSTAT3 of 3.1 ± 0.5 and 3.6 ± 0.4-fold, respectively. Results represent means ± S.E. (error bars) from eight independent experiments. *, p < 0.05 compared with unstimulated controls.

**Figure 3.** Synergistic activation of phosphorylated STAT3 and PI3K by subthreshold doses of Leptin/CCK-8 in rat NG. A, representative immunoblot of pSTAT3 and actin in acutely isolated rat NG stimulated for 10 min with 0.01 nM leptin, 0.01 nM CCK-8, or a combination of 0.01 nM leptin and 0.01 nM CCK-8 is shown. B, combination of 0.01 nM leptin and 0.01 nM CCK-8 synergistically stimulated pSTAT3 by 3.6 ± 0.26-fold, whereas leptin or CCK-8 alone failed to stimulate pSTAT3. C, representative immunoblot of pPI3K and actin in acutely isolated rat NG stimulated for 10 min with 0.01 nM leptin, 0.01 nM CCK-8, or a combination of 0.01 nM leptin and 0.01 nM CCK-8 is shown. D, combination of 0.01 nM leptin and 0.01 nM CCK-8 synergistically stimulated phosphorylated PI3K by 3.1 ± 0.56-fold, whereas leptin or CCK-8 alone failed to stimulate pPI3K. The data represent means ± S.E. (error bars) from three independent experiments. *, p < 0.05 compared with the response to control, CCK-8, or leptin alone.
FIGURE 4. Leptin/CCK-8 synergistic activation of phosphorylated STAT3 in rat NG. A, representative immunoblot of pSTAT3, STAT3, and actin in acutely isolated rat NG stimulated for 10 min with 1 nM leptin, 1 nM CCK-8, or a combination of 1 nM leptin and 1 nM CCK-8 is shown. Note that 1 nM CCK-8 and leptin each caused an increase in STAT3. B, combination of 1 nM leptin and 1 nM CCK-8 synergistically stimulated pSTAT3 by 7.68 ± 0.7-fold, which was significantly higher than that evoked by leptin (1 nM) or CCK-8 (1 nM) alone. The data represent means ± S.E. (error bars) from five independent experiments. *, p < 0.05 compared with unstimulated controls; **, p < 0.05 compared with the response to CCK-8 or leptin alone.

increase in pPI3K over the basal level. A combination of 0.01 nM CCK-8 and 0.01 nM leptin caused a synergistic increase PI3K phosphorylation as demonstrated in a representative immunoblot (Fig. 3C). 0.01 nM CCK-8 and 0.01 nM leptin increased PI3K phosphorylation by 3.1 ± 0.56-fold, which was significantly higher than the basal level of pPI3K (p < 0.05, Fig. 3D). We also tested the activation of maximal doses of CCK-8 and leptin. 1 nM CCK-8 and 1 nM leptin resulted in a synergistic increase in pSTAT3 and actin, 1 nM CCK-8 and 1 nM leptin alone caused peak increases in pSTAT3 of 2.5 ± 0.5 and 2.9 ± 0.41, respectively (Fig. 4B). A combination of 1 nM CCK-8 and 1 nM leptin increased STAT3 phosphorylation by 7.68 ± 0.7-fold, which was significantly higher than the maximal increase in pSTAT3 evoked by CCK-8 or leptin alone (p < 0.05, Fig. 4B). These findings indicate that the interaction between CCK-8 and leptin is potentiation rather than addition.

Effect of Inhibitors on Leptin/CCK-8 Synergistic Phosphorylation of STAT3 and PI3K—To determine the downstream signaling pathways involved in the CCK-8 and leptin synergism, we examined the effect of inhibitors on STAT3 phosphorylation evoked by 1 nM leptin plus 1 nM CCK-8 because these concentrations caused a maximal increase in pSTAT3 and pPI3K. A representative pSTAT3 and actin immunoblot shows the effect of the inhibitors (Fig. 5A). The leptin/CCK-8 pSTAT3 synergism caused a 7.25 ± 0.81-fold increase in pSTAT3 and this was inhibited 62.8% by the RhoA inhibitor C3 transferase (0.5 μg/ml), 65.3% by the Src kinase inhibitor PP2 (10 μM), and 60.7% and 64.4%, respectively, by the PI3K inhibitors LY294002 (50 μM) and wortmannin (1 μM) (p < 0.05). The JAK2 inhibitor AG490 (50 μM) inhibited STAT3 phosphorylation by 99.2% (p < 0.05) (Fig. 5B). In contrast, the MAPK inhibitor PD98059 (50 μM) had no effect on the synergistic action of leptin and CCK-8. The doses used for each antagonist have been shown previously to be effective in cell culture systems. To elucidate further the role of PI3K in the leptin/CCK synergism, we studied the effect of 1 nM leptin or 1 nM CCK-8 alone and in combination on PI3K phosphorylation. A representative phosphorylated PI3K (pPI3K) immunoblot (Fig. 6A) shows the synergistic increase in pPI3K in response to a combination of 1 nM leptin and 1 nM CCK-8. In combination, these peptides caused a 5.05 ± 0.95-fold increase in PI3K phosphorylation (p < 0.05, Fig. 6B). The synergistic increase in pPI3K evoked by 1 nM leptin and 1 nM CCK-8 was inhibited 84.2% by C3 transferase (0.5 μg/ml), 73.3% by PP2 (10 μM), and 84.01% by LY294002 (50 μM). In addition, AG490 (50 μM) inhibited PI3K phosphorylation by 93.7% (Fig. 6B). Our data suggest that PI3K may be central for the synergistic interaction between CCK-8 and leptin. CCKAR activation stimulates Src via protein kinase C, which leads to phosphorylation of PI3K. Meanwhile, activation of LRb also stimulates PI3K via stimulation of JAK2 and the insulin receptor substrate (see Fig. 9).

Silencing PI3K and Src Genes Abolishes Leptin/CCK Synergistic Phosphorylation of STAT3—To provide conclusive evidence that PI3K and Src are involved in the phosphorylation of STAT3, we silenced the Src and PI3K genes. Primary cultured NG neurons were transiently transfected for 3 days with PI3K siRNA or Src siRNA and then stimulated for 10 min with and without a combination of 1 nM leptin and 1 nM CCK-8. Representative pSTAT3, PI3K, Src, and actin immunoblots show the effects of scrambled siRNA, PI3K siRNA (Fig. 7A), and Src siRNA (Fig. 7B). Transfection of NG neurons with PI3K siRNA
or Src siRNA caused a significant decrease in total PI3K and total Src expression (>60%) and resulted in a loss of leptin/CCK synergistic phosphorylation of STAT3, whereas transfection with scrambled PI3K siRNA or scrambled Src siRNA did not affect PI3K and Src expression and the synergistic interaction of leptin/CCK-8, which caused a 6.3 ± 0.6 and 7.5 ± 0.7-fold increase in pSTAT3, respectively (Fig. 7, A and B). On the other hand, transient transfection of cultured rat NG neurons with ERK1/ERK2 siRNA inhibited MAPK by 63.6% (Fig. 7C). However, inhibition of MAPK by ERK1/ERK2 siRNA had no effect on STAT3 phosphorylation or total STAT3 in cultured rat NG neurons (Fig. 7C). These data suggest that both Src and PI3K, but not MAPK, play a major role in the production of pSTAT3 evoked by leptin/CCK-8 synergism in the rat NG. Transfection efficiency with siRNA for PI3K, Src, and MAPK was >70%.

**Silencing the STAT3 Gene Abolishes Leptin/CCK Neuronal Firing**—We also examined the effect of silencing the STAT3 gene on neuronal firing of the rat NG. Cultured rat NG neurons were infected with lentiviral STAT3-shRNA for 5 days to abolish STAT3. NG infected with lentiviral STAT3-shRNA were identified by GFP labeling. We performed patch clamp studies on NG neurons transfected with STAT3-shRNA. Continuous membrane potential recordings of cultured NG neurons infected with lentiviral STAT3-shRNA for 5 days showed no change in input resistance with either leptin or CCK-8 alone or combined in the 12 NG neurons tested (p < 0.05) (Fig. 8A) in three independent experiments. Immunoblots verified that there was little or no STAT3 (Fig. 8B). Western immunoblotting showed a loss of the leptin/CCK-8 synergistic phosphorylation of STAT3 in NG neurons infected with STAT3-shRNA, compared with NG infected with scrambled lentiviral shRNA, which showed a 7.5 ± 0.2-fold increase in STAT3 phosphorylation (Fig. 8B). A GFP-labeled neuron (Fig. 8C) showed that the recordings were performed in successfully infected neurons. Transfection efficiency with the retroviral STAT3-shRNA was 95 ± 3% in these neurons. Current recordings showed that the cells were spiking and that normal membrane properties were not affected (Fig. 8D). Our data suggest that leptin/CCK-8-stimulated phosphorylation of STAT3 was responsible for the enhanced neuronal firing.

**DISCUSSION**

In this study, we showed a synergistic interaction between leptin and CCK-8 in whole cell patch clamp recordings of neurons isolated from rat NG, resulting in an increase in membrane input resistance, which was significantly larger than the increase in membrane resistance evoked by CCK-8 or leptin alone (Fig. 1). We also observed a synergistic phosphorylation of STAT3 by a combination of leptin and CCK-8 in Western immunoblots of acutely isolated NG neurons, which was significantly different from the response to leptin or CCK-8 alone (Figs. 3 and 4). Previous studies reported a similar synergistic interaction between leptin and CCK in cultured NG neurons.
Leptin and Cholecystokinin Synergistic Interaction

(24, 29); however, the downstream signaling pathways involved in the leptin/CCK-8 synergism and its effect on neuronal firing have not been elucidated. We hypothesized that STAT3 may play a pivotal role in the leptin/CCK-8 synergism. Previous studies reported that leptin directly activates STAT3 phosphorylation (30, 31). Our results showed that silencing the STAT3 gene with lentiviral shRNA abolished the synergistic action of leptin and CCK-8 (Fig. 8) and completely inhibited continuous membrane potential recordings in response to leptin and CCK-8, suggesting that pSTAT3 regulates the closure of K\(^{+}\) channels.

To understand the downstream signal transduction pathways involved in the leptin/CCK-8 synergism, we examined the effect of inhibitors on the synergistic activation of STAT3 phosphorylation in acutely isolated NG neurons. Our studies showed that the synergistic leptin/CCK-8-stimulated phosphorylation of STAT3 was inhibited by the JAK2 inhibitor AG490, the RhoA inhibitor C3 transferase, the Src kinase inhibitor PP2, and the PI3K inhibitors LY294002 and wortmannin (Fig. 5). Activation of CCK-A and leptin receptors may stimulate MEK1/2, resulting in phosphorylation of ERK1/2 (Fig. 7). However, the MAPK inhibitor PD98059, or ERK1 and ERK2 siRNA transfection, had no effect on leptin/CCK-8 synergism, indicating that the leptin/CCK-8-stimulated MAPK/ERK1/2 pathway was not involved in STAT3 phosphorylation. Previous studies have reported that CCK-8 activation of ERK signaling leading to phosphorylation of the Kv4.2 potassium channel in rat nucleus of the solitary tract may be involved in the short term suppression of food intake in rats (32). However, this pathway is not involved in mediating the synergistic interaction of leptin/CCK-8 in the NG neurons.

It is well established that central administration of leptin enhanced the satiety action of peripheral CCK (33) and increased the number of labeled nucleus of the tractus solitarius neurons (34). Because leptin was administered centrally, it is quite conceivable that rat vagal afferent negative feedback signals evoked by CCK and central adiposity signals from leptin may be integrated at the nucleus of the tractus solitarius (35). However, this does not exclude the possibility that CCK and leptin may also interact synergistically in the NG. In fact, it has been demonstrated that perivagal capsaicin abolished the synergistic interaction between leptin and CCK to reduce short term feeding (23). We propose that the synergistic interaction between CCK and leptin may occur at multiple levels along the vagovagal pathways. The primary site may be the NG, which are very sensitive to these peptides as a synergistic increase in pSTAT3 may be observed with 0.01 nM leptin and CCK. Considering that these peptides may be acting locally on vagal afferent fibers innervating the duodenal mucosa, these concentrations may be within physiological ranges.

FIGURE 8. Silencing the STAT3 gene inhibited leptin/CCK-8-stimulated neuronal firing. A, representative continuous membrane potential recordings in response to extracellular application of 1 nM leptin or 1 nM CCK-8 or a combination of 1 nM leptin and 1 nM CCK-8 in a patch clamp study of cultured NG neurons, 5 days after infection with STAT3-shRNA. B, representative immunoblot for pSTAT3, STAT3, and actin in cultured rat NG neurons after a 5-day infection with STAT3-shRNA, and then stimulated for 10 min with a combination of 1 nM leptin and 1 nM CCK-8. C, GFP-labeled neuron showing that the recordings were performed in successfully infected NG neurons. D, current recordings in the GFP-labeled neuron showing that the cells were spiking and normal membrane properties were not affected. The data are representative of three independent experiments.
Leptin has been considered a long term regulator of nutrient intake, adiposity, and body weight (5) whereas CCK-8 acts as a meal-related short term satiety signal (19, 20). The satiety signal generated by CCK-8 is relatively weak. However, the synergistic interaction between leptin and CCK-8 significantly amplifies the signal and enhances the ability of leptin to act not only as a regulator of long term feeding behavior and energy balance, but also to serve as a mediator of short term satiety. In this manner, CCK-8 acting on low affinity vagal CCKAR (16) serves as a facilitator, rather than a primary initiator, to induce satiety. On the other hand, leptin, by interacting with CCK-8, becomes a major mediator to control short term food intake, as well as a regulator for long term feeding behavior and body weight homeostasis (23). In this study, we delineated the intracellular mechanisms by which CCK-8 interacts with leptin to enhance NG excitation through STAT3 signaling.

STAT3 usually acts by stimulating the transcription of target genes (33), but the rapid electrophysiological effects that were observed in this study and that have been reported by others (24, 29, 34) are not likely to be explained by STAT3-mediated transcription. It is possible that STAT3 may be involved in modifying the activity of K^+ channels. Until now, leptin has been considered a long term regulator of nutrient intake, adiposity, and body weight (5) whereas CCK-8 acts as a meal-related short term satiety signal (19, 20). The satiety signal generated by CCK-8 is relatively weak. However, the synergistic interaction between leptin and CCK-8 significantly amplifies the signal and enhances the ability of leptin to act not only as a regulator of long term feeding behavior and energy balance, but also to serve as a mediator of short term satiety. In this manner, CCK-8 acting on low affinity vagal CCKAR (16) serves as a facilitator, rather than a primary initiator, to induce satiety. On the other hand, leptin, by interacting with CCK-8, becomes a major mediator to control short term food intake, as well as a regulator for long term feeding behavior and body weight homeostasis (23). In this study, we delineated the intracellular mechanisms by which CCK-8 interacts with leptin to enhance NG excitation through STAT3 signaling. STAT3 usually acts by stimulating the transcription of target genes (33), but the rapid electrophysiological effects that were observed in this study and that have been reported by others (24, 29, 34) are not likely to be explained by STAT3-mediated transcription. It is possible that STAT3 may be involved in modifying the activity of K^+ channels.

Our studies showed that CCK and leptin interact synergistically to enhance neural excitability of the NG. Other studies reported that CCK interacts with leptin at the level of vagal afferent neurons to control the function of the early growth response factor-1 (EGR1) (36). Leptin stimulates EGR1 expression whereas CCK stimulates redistribution to the nucleus of EGR1 in the nodose ganglia neuron. Through this mechanism, CCK and leptin interact cooperatively to regulate the expression of the gene encoding the satiety peptide cocaine- and amphetamine-regulated transcript (CARTp) via an EGR1-dependent mechanism (36). Therefore CCK and leptin may interact at multiple levels in the nodose ganglia to generate satiety signaling to inhibit feeding.

We conclude that the synergistic interaction between the vagal CCKAR and the leptin receptor is mediated by the phosphorylation of STAT3, which, in turn, activates the closure of K^+ channels, leading to membrane depolarization and neuronal firing in rat NG. The leptin/CCK-8 synergism involves the interaction between CCK/Src/PI3K cascades and the leptin/JAK2/PI3K/STAT3 signaling pathways, with a major role for PI3K. As CCKARs and LRbs are widely distributed in the NG (21), and leptin and CCK play a role in regulating satiety (23), it is conceivable that malfunctioning of these signaling molecules may result in eating disorders.

Acknowledgments—We thank the Retroviral Core Laboratory at the University of Michigan for the providing the lentiviral shRNA which was produced with the assistance of Thomas Lanigan.

REFERENCES

1. Maffei, M., Halaas, J., Ravussin, E., Pratley, R. E., Lee, G. H., Zhang, Y., Fei, H., Kim, S., Lallone, R., and Ranganathan, S. (1995) Nat. Med. 1, 1155–1161
2. Loftus, T. M. (1999) Semin. Cell Dev. Biol. 10, 11–18
3. Pan, W., and Kastin, A. J. (2001) Life Sci. 68, 2705–2714
4. Lee, G. H., Proenca, R., Montez, J. M., Carroll, K. M., Darvishzadeh, J. G., Lee, J. I., and Friedman, J. M. (1996) Nature 379, 632–635
5. Fei, H., Okano, H. J., Li, C., Lee, G. H., Zhao, C., Darnell, R., and Friedman, J. M. (1997) Proc. Natl. Acad. Sci. U.S.A. 94, 7001–7005
6. Masuzaki, H., Ogawa, Y., Sagawa, N., Hosoda, K., Matsumoto, T., Mise, H., Nishimura, H., Yoshimasa, Y., Tanaka, I., Mori, T., and Nakao, K.
(1997) Nat. Med. 3, 1029–1033
7. Sobhani, I., Bado, A., Vissuzaine, C., Buyse, M., Kermorgant, S., Laigneau, J. P., Attoub, S., Lehy, T., Henin, D., Mignon, M., and Lewin, M. J. (2000) Gut 47, 178–183
8. Bado, A., Levasseur, S., Attoub, S., Kermorgant, S., Laigneau, J. P., Bor-
toluzzi, M. N., Moizo, L., Lehy, T., Guerre-Millo, M., Le Marchand-Brus-
tel, Y., and Lewin, M. J. (1998) Nature 394, 790–793
9. Zhang, Y., Proenca, R., Maffei, M., Barone, M., Leopold, L., and Friedman, J. M. (1994) Nature 372, 425–432
10. Björnholm, M., Münzberg, H., Leshan, R. L., Villanueva, E. C., Bates, S. H., Louis, G. W., Jones, J. C., Ishida-Takahashi, R., Bjørbaek, C., and Myers, M. G., Jr. (2007) J. Clin. Invest. 117, 1354–1360
11. Montague, C. T., Farooqi, I. S., Whitehead, J. P., Soos, M. A., Rau, H., Wareham, N. J., Sewter, C. P., Digby, J. E., Mohammed, S. N., Hurst, J. A., Cheerham, C. H., Earley, A. R., Barnett, A. H., Prins, J. B., and O’Rahilly, S. (1997) Nature 387, 903–908
12. White, D. W., Kuropatwinski, K. K., Devos, R., Baumann, H., and Tarta-
glia, L. A. (1997) J. Biol. Chem. 272, 4065–4071
13. Banks, A. S., Davis, S. M., Bates, S. H., and Myers, M. G., Jr. (2000) Am. J. Physiol. Regul. Integr. Comp. Physiol. 279, R1238–1246
14. Jiang, L., Li, Z., and Rui, L. (2008) J. Biol. Chem. 283, 28066–28073
15. Crawford, J. N., and Corwin, R. L. (1994) Peptides 15, 731–755
16. Weatherford, S. C., Laughton, W. B., Salabarria, J., Danho, W., Tilley, J. W., Netterville, L. A., Schwartz, G. J., and Moran, T. H. (1993) Am. J. Physiol. Regul. Integr. Comp. Physiol. 264, R244–249
17. Baldwin, B. A., Parrott, R. F., and Ebenezer, I. S. (1998) Prog. Neurobiol. 55, 477–507
18. Passaro, F. Jr., Debas, H., Oldendorf, W., and Yamada, T. (1982) Brain Res. 241, 335–340
19. Moran, T. H., Ameglio, P. J., Schwartz, G. J., and McHugh, P. R. (1992) Am. J. Physiol. Regul. Integr. Comp. Physiol. 262, R46–R50
20. Ritter, R. C., Covasa, M., and Matson, C. A. (1999) Neuropeptides 33, 387–399
21. Burdyga, G., Spiller, D., Morris, R., Lal, S., Thompson, D. G., Saeed, S., Dimaline, R., Varro, A., and Dockray, G. J. (2002) Neuroscience 109, 339–347
22. Zarbin, M. A., Wamsley, J. K., Innis, R. B., and Kuhar, M. J. (1981) Life Sci. 29, 697–705
23. Barrachina, M. D., Martínez, V., Wang, L., Wei, J. Y., and Taché, Y. (1997) Proc. Natl. Acad. Sci. U.S.A. 94, 10455–10460
24. Peters, J. H., Karpel, A. B., Ritter, R. C., and Simasko, S. M. (2004) Endo-
crinology 145, 3652–3657
25. Williams, D. L., Baskin, D. G., and Schwartz, M. W. (2009) Am. J. Physiol. Regul. Integr. Comp. Physiol. 297, R1238–1246
26. Owyang, C., Hao, Y., and Li, Y. (2008) Gastroenterology 134, Suppl. 1, A-96–A-97 (abstr.)
27. Tsunoda, Y., Yoshida, H., Africa, L., Steil, G. J., and Owyang, C. (1996) Biochem. Biophys. Res. Commun. 227, 876–884
28. Nozu, F., Owyang, C., and Tsunoda, Y. (2000) Eur. J. Cell Biol. 79, 803–809
29. Peters, J. H., Ritter, R. C., and Simasko, S. M. (2006) Am. J. Physiol. Cell Physiol. 290, C427–432
30. Frontini, A., Bertolotti, P., Tonello, C., Valerio, A., Nisoli, E., Cinti, S., and Giordano, A. (2008) Brain Res. 1215, 105–115
31. Ruiter, M., Duffy, P., Simasko, S., and Ritter, R. C. (2010) Endocrinology 151, 1509–1519
32. Sutton, G. M., Patterson, L. M., and Berthoud, H. R. (2004) J. Neurosci. 24, 10240–10247
33. Zhang, X., Wrzeszczynska, M. H., Horvath, C. M., and Darnell, J. E., Jr. (1999) Mol. Cell. Biol. 19, 7138–7146
34. Wang, Y. H., Taché, Y., Sheibel, A. B., Go, V. L., and Wei, J. Y. (1997) Am. J. Physiol. Regul. Integr. Comp. Physiol. 273, R833–837
35. Grill, H. J. (2010) Front. Neuroendocrinol. 31, 61–78
36. de Lartigue, G., Lur, G., Dimaline, R., Varro, A., Raybould, H., and Dock-
ray, G. J. (2010) Endocrinology 151, 3589–3599