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Modulation of food intake by mTOR signaling in the dorsal motor nucleus of vagus in male rats: Focus on ghrelin and nesfatin-1

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Abstract

Previous studies demonstrate that mTOR signaling in the hypothalamus is involved in the control of energy homeostasis. The aim of this study is to characterize the effect of mTOR signaling in the dorsal motor nucleus of vagus (DMNV) on the energy intake. Phosphor-mTOR was detected in the DMNV neurons and its levels increased by energy deprivation. Rapamycin significantly inhibited mTOR activity and reduced food intake when administrated into 4th ventricle. Exposure of DMNV neurons to ghrelin increased the phosphorylation of mTOR. Fourth ventricle injection of ghrelin significantly increased food intake relative to the control vehicle. Pre-treatment with rapamycin for 15 min attenuated the orexigenic effect of ghrelin. Reduction in the phosphorylation of mTOR was observed following 4th intracerebroventricular injection of nesfatin-1. When administrated by 4th ICV injection, nesfatin-1 suppressed the food intake as compared with the control. The anorexigenic effect of nesfatin-1 was significantly attenuated by pre-treatment with leucine for 15 min. All these studies suggest that mTOR signaling in the DMNV neurons regulates both the nutrient and hormonal signals for the modulation of food intake.

Keywords

Dorsal vagal complex; ghrelin; nesfatin-1

Introduction

mTOR, a highly conserved serine-threonine kinase, has been reported to serve as an intracellular ATP sensor (Dennis et al., 2001; Inoki et al., 2005). In vitro studies have demonstrated that cellular levels of ATP regulate mTOR signaling (Dennis et al., 2001; Inoki et al., 2005). Aberrant mTOR activity is linked to diabetes and obesity, and significant elevation of mTOR signaling has been observed in liver and skeletal muscle of insulin-resistant obese rats maintained on a high fat diet (Khamzina et al., 2005). In contrast, absence of the mTOR downstream target, S6 kinase 1, protects against diet-induced obesity and improves insulin sensitivity in mice (Um et al., 2004). Gastric mTOR has been demonstrated to be critical for the production of two important gastric hormones derived from the X/-like endocrine cells, ghrelin (Xu et al., 2010; Xu et al., 2009) and nesfatin-1 (Li et al., 2012), which modulate food intake (Nakazato et al., 2001; Oh-I et al., 2006; Stengel et

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Disclosures
The authors have nothing to disclose.

al., 2009; Stengel et al., 2012; Tschop et al., 2000; Xu et al., 2010) and glucose homeostasis (Sun et al., 2006; Xu et al., 2012; Yang et al., 2010).

mTOR signaling in hypothalamic neurons is involved in neuronal sensing of nutrient availability, and regulates food intake and energy balance, suggesting that mTOR signaling in the central nervous system is crucial for the regulation of energy metabolism (Cota et al., 2006; Mori et al., 2009). It is currently unknown whether mTOR signaling is present in the dorsal vagal complex, a group of nuclei critical for the coordination of gastrointestinal functions (Browning et al., 2011; Travagli et al., 2006; Zheng et al., 2005). The dorsal vagal complex (DVC), a paired structure located in the dorsal, caudal medulla alongside the central canal which is contiguous with the 4th ventricle, is comprised of three adjacent and functionally integrated nuclei: the NTS, the area postrema and the dorsal motor nucleus of the vagus (DMNV). The DVC is a critical relay in vagal-vagal circuits. Afferent vagal fibers from the gastrointestinal tract synapse on interneurons located within the NTS. These neurons synapse on efferent vagal motor neurons in the DMNV. Pre-ganglionic efferent vagal fibers project to ganglia in the upper gastrointestinal tract, providing parasympathetic control of many aspects of gastrointestinal function, including motility, secretion, and absorption (Browning et al., 2011; Travagli et al., 2006; Zheng et al., 2005).

While fuel sensing molecules such as AMP-activated kinase (AMPK) (Lam et al., 2011; Minokoshi et al., 2004; Yang et al., 2010) and mTOR (Cota et al., 2006; Mori et al., 2009) have been implicated in the coordination of energy supplies, food intake and energy expenditure in the hypothalamic neurons, their roles in the dorsal vagal complex are less well studied. Previous reports have demonstrated that AMPK activity in the hindbrain contributes to control of energy balance through regulation of food intake and energy expenditure (Hayes et al., 2009). The present study examines mTOR signaling in the dorsal vagal complex. We report that alterations in mTOR signaling in the DMNV in response to fasting and the gastric hormones ghrelin and nesfatin-1 are involved in the regulation of food intake.

Methods

Ethical approval

The animals used in this study were handled in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996), and all the experimental protocols (08402) were approved by the University of Michigan Committee on the Use and Care of Animals.

Chemicals and solutions

Neurobasal medium, phosphate buffer solution (PBS), B27 supplement, L-glutamine, penicillin and streptomycin were purchased from Gibco (Grand Island, NY). β fibroblast growth factor (βFGF) was from Invitrogen (Carlsbad, CA). Poly-L-lysine and Triton X-100 were purchased from Sigma-Aldrich (St. Louis, MO). Ghrelin and nesfatin-1 peptides were from Phoenix Pharmaceuticals (Burlingame, CA). Rabbit anti-phospho-mTOR (Ser2448) polyclonal antibody, phosphor-mTOR (Ser2448) and mTOR mouse monoclonal antibodies, phosphorylated ribosomal protein S6 (pS6; ser235/236) mouse antibody, S6 ribosomal mouse antibody were from Cell Signaling Technology (Beverly, MA). The following secondary antibodies: FITC-conjugated goat anti-mouse IgG and TRITC-conjugated goat anti-rabbit IgG were obtained from Jackson ImmunoResearch (West Grove, PA). Intracerebroventricular cannulas were purchased from Plastics One (Roanoke, VA) and cut to 7.4 mm.

Animal Care

Animals were housed in a temperature controlled environment with 12 h light and dark cycles, and access to food and water ad libitum except indicated.

4th Ventricle Cannulation

Male Sprague-Dawley rats weighing 200–250 g were anesthetized with intra-peritoneal injection of a mixture of xylazine and ketamine (13 and 87 mg/kg body weight, respectively). A 26G single lumen cannula was introduced into the 4th ventricle stereotactically according to coordinates published by Paxinos and Watson: midline, 13 mm caudal to bregma, and 7.4 mm deep with a head tilt of 0:−3.3 mm13. Screws were placed into the skull surrounding the cannula, and the cannula secured with craniofacial cement. Finally, a sterile internal cannula was placed to maintain patency and prevent infection. On post-procedure day 3, cannula position was tested by 4th intracerebroventricular (ICV) injection of 5-thio-D-glucose, 210 μg in 3 μl PBS. A greater than 50% rise in blood glucose in response to 5-thio-D-glucose was considered confirmation of correct cannula position. On post-procedure day 7, animals were acclimated and mock-injected with 2μL of CFS. On post-procedure day 9, animals underwent 2 μL injection of CFS, rapamycin, leucine, ghrelin, nesfatin-1 or artificial CSF over one minute period either at 6:00 pm in the dark phase or 8:00 am in the light phase. The doses of ghrelin and nesfatin-1 were chosen based on published literature (Nakazato et al., 2001; Oh-I et al., 2006) and previous experiments in our laboratory (Zhang et al., 2001). Rats were group-housed and food intake in ad libitum fed animals was measured before and after ICV injection.

Neuronal culture of dorsal motor nucleus

Dorsal motor nucleus neurons were isolated from neonatal Sprague-Dawley rats (Charles River, Wilmington, MA) as described previously (Zhang et al., 2004; Zhang et al., 2006). The procedures used for the care and euthanasia of the animals were approved by the University of Michigan Committee on Use and Care of Animals. Briefly, rats were euthanized by CO_2 inhalation followed by bilateral pneumothorax. The brainstem was rapidly removed and chilled at 0°C in a dissection solution containing: NaCl 138 mM, KCl 4 mM, $MgCl_2$ 1 mM, $CaCl_2$ 2 mM, glucose 20 mM and HEPES 10 mM. Tissue blocks were prepared and sectioned transversely into 400 μm slices at the level of the obex using a Vibratome 3000 (Redding, CA). The DMNV area was identified under a dissecting microscope as the area immediately ventral to the nucleus of the solitary tract and dorsal to the XII nucleus. DMNV tissue was excised and then digested in an enzyme solution containing protease type XIV (0.6 mg/ml) and trypsin type I (0.4 mg/ml) at 32 °C for 30 minutes. The tissue was then dissociated by gentle trituration with pipettes. Cells were plated onto poly-L-lysine coated culture dishes and chamber slides. Neurons were maintained at 37°C in an atmosphere of 5% CO2 in serum-free culture media containing Neurobasal medium containing 2% B27 supplement, 2 mM glutamine, 1% penicillin and streptomycin, and 5 ng/ml βFGF. After 4 days, one-half of the medium was replaced and experiments were conducted at 7 days.

Immunofluorescence

Animals were deeply anesthetized by an intramuscular injection of sodium pentobarbital at a dose of 75 mg (kg body weight)$^{-1}$ and sacrificed. Intra-cardiac perfusion with 4% paraformaldehyde, preparation of tissue blocks, and sectioning at a thickness of 10μm was performed as described previously (Zhang et al., 2004). Slides at the level of area postrema were first washed in PBS for 30 minutes, then incubated with a blocking solution (10% normal goat serum, 3% BSA, and 0.2% Triton X-100 in PBS, pH7.4) for 1 h at room temperature. Sections were then treated with primary antibody or control IgG at 4°C for 24

h, washed in PBS, and treated with secondary antibody for 1 h at room temperature. Finally, slides were incubated with DAPI (1:5000) and mounted with ProLong Gold anti-fade reagent. All antibodies were diluted in blocking solution. Primary antibodies utilized include mouse anti-Hu (1:100), rabbit anti-pmTOR (1:100). Control antibodies used mouse or rabbit IgG. TRITC-conjugated goat anti-rabbit IgG (1:100) and FITC-conjugated goat anti-mouse IgG (1:100) were used as secondary antibodies. Slides were viewed using a fluorescent microscope (Nikon Eclipse Ti-U) (Nikon Inc., Melville, NY). The DMNV was identified as described above. Positive signal was identified and calculated using the following criteria: Relative optical densities were established for the regions of interest (ROI) by Image-Pro Plus (Media Cybernetics Inc., Silver Spring, MD). Results were expressed as the difference in gray-scale levels comparing ROI and the background regions in the same section. A change of 100% and higher in optical density was used as the threshold for positive cells. Four sections from the same animal were counted and averaged. Neurons stained positively for phosphor-mTOR were counted and expressed as percentage of Hu-positive cells. Results are expressed as mean±SEM.

Western blotting

After treatment, DMNV neuronal cultures were homogenized in 60μl ice-cold lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na_3VO_4, 1 μg ml^{-1} leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF), then centrifuged at 12,000 g at 4°C for 20 min. Equal amounts of protein (30~40μg) from each sample were subjected to electrophoretic separation on a 10% polyacrylamide gel (BioRad, Hercules, CA), then transferred onto Immobilon™ PVDF membrane (BioRad). Membrane blots were blocked at room temperature for 1 h in 5% milk in TBS–Tween 20 (0.05%), then incubated overnight in primary antibodies against mTOR, or phosphor-mTOR (1:1,000 dilution) from Cell Signaling Technology (Danvers, MA). Membranes were washed three times in TBS–Tween (0.05%), then incubated for 1 h with secondary antibody diluted 1/4000 in 5% TBS–Tween (0.05%). Detection was performed using Lumi-Light Blotting Substrate (Roche, Indianapolis, IN) or SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific, Rockford, IL).

Data analysis

Results are expressed as mean ± SEM. Data were analyzed using ANOVA and Student's t-test as appropriate. Significance was accepted as $P < 0.05$.

Results

mTOR signaling in the DVC

As shown in Figure 1a, phosphor-mTOR was detected in the majority of DMNV neurons and a few NTS neurons, suggesting the presence of mTOR signaling in the DVC. No positive signal was observed in the control study. Since mTOR signaling was detected mainly in the DMNV neurons, we focused our study on this nucleus instead of the NTS. We first examined alterations in phosphor-mTOR in DMNV neurons in response to fasting. As shown in Figure 1b, fasting for 24 h significantly increased the phosphorylation of mTOR in Hu (a neuronal marker) positive DMNV neurons relative to the fed animals (81±6% in fasted mice vs 14±9% in fed animals, $P<0.01$), suggesting an increase in the mTOR signaling.

Regulation of food intake by mTOR signaling in DMNV neurons

If mTOR signaling contributes to energy homeostasis, one would expect that inhibition of mTOR signaling in the hindbrain would alter food intake. Rapamycin, a specific inhibitor of mTOR signaling, was used in the following experiments to verify this concept. We first examined the effect of rapamycin on mTOR signaling in primary DMNV cultures. Cells were incubated with different concentrations of rapamycin for 30 min. As shown in Figure 2a, rapamycin at the doses of 5 and 50 nM significantly decreased levels of phosphor-mTOR, suggesting inhibition of mTOR signaling in cultured DMNV neurons.

To examine the effect of mTOR signaling in the DVC on food intake, rats were treated with 4th ICV injection of either rapamycin at a dose of 50 μg in 2 μl or vehicle. As shown in Figure 2b, relative to the control rats receiving vehicle administration, animals treated with 4th ICV injection of rapamycin demonstrated a significant decrease in food intake, with persistent effects lasting for up to three days.

Effects of ghrelin on mTOR signaling and food intake

Gastric hormones such as ghrelin and nesfatin-1 have been reported to regulate food intake by a central mechanism. We next examined whether mTOR signaling in the DMNV is involved in ghrelin-induced stimulation of food intake. As shown in Figure 3a, treatment of cultured DMNV neurons with 20 and 200 nM of ghrelin for 30 min significantly increases levels of phosphor-mTOR. The doses of ghrelin are chosen based on the literature and previous experiments in our laboratory. Fourth ICV injection of ghrelin significantly increased the phosphorylation of mTOR in the DMNV neurons stained positively for Hu, a neuronal marker (Figure 3b), (61±14% vs. 22±10% of control, $P<0.05$). Administration of 5 and 10 μg ghrelin into the 4th ventricle significantly increased the food intake during the light cycle (Figure 3c). Pretreatment with rapamycin (37.5 μg) for 15 min attenuated the orexigenic effect of 10 μg ghrelin (Figure 3d). These results suggest that ghrelin stimulates food intake through activation of mTOR signaling in DMNV neurons.

Effects of nesfatin-1 on mTOR signaling and food intake

The effect of nesfatin-1, a gastric anorexigenic hormone, on mTOR signaling in the DMNV neurons and food intake was next investigated. As shown in Figure 4a, cultured DMNV neurons exposed to 0.1 and 1 nM of nesfatin-1 for 30 min demonstrated a significant decrease in levels of phosphor-mTOR, suggesting an inhibition of mTOR signaling. Similar reduction in levels of phosphor-mTOR was observed in DMNV neurons when exposed to nesfatin-1 by 4th ICV injection (Figure 4b), (4±5% vs. 22±10% of control, $p<0.05$). Nesfatin-1 (0.2 and 2 μg) administrated by 4th ICV injection markedly reduced the food intake during the dark cycle (Figure 4c). Pretreatment with leucine (1 μg), a branched-chain α amino acid with potent agonism of mTOR signaling, for 15 min significantly attenuated the anorexigenic effect of 2 μg nesfatin-1 (Figure 4d).

Combined effect of ghrelin and nesfatin-1 on mTOR signaling and food intake

The combined effect of ghrelin and nesfatin-1 were further examined. As shown in figure 5a, simultaneous administration of ghrelin (5 μg) and nesfatin-1 (2 μg) significantly attenuated the activation of mTOR signaling in DMNV neurons relative to ghrelin alone (Figure 5a), accompanied by a marked reduction in food intake relative to ghrelin treatment alone (Figure 5b).

Discussion

The current study demonstrates that mTOR signaling in DMNV neurons is involved in the regulation of food intake. This general conclusion is supported by four distinct observations:

(1) mTOR signaling is present in DMNV neurons and its activity is up-regulated by fasting; (2) Inhibition of mTOR signaling by rapamycin decreases food intake; (3) Ghrelin administrated by 4th ICV injection markedly increases food intake. This effect is mediated by activation of mTOR signaling in DMNV neurons; (4) In contrast, nesfatin-1 decreases food intake through inhibition of mTOR signaling in DMNV neurons. (5) Simultaneous administration of ghrelin and nesfatin-1 blunts increases in mTOR signaling and food intake induced by ghrelin.

mTOR signaling in the hypothalamus is known to be involved in the regulation of food intake and energy expenditure (Cota et al., 2006; Mori et al., 2009). Whether extrahypothalamic mTOR signaling in the central nervous system contributes to the control of energy homeostasis has not been previously demonstrated. Here we demonstrate that mTOR signaling in DMNV neurons is responsive to energy status and to both ghrelin and nesfatin-1, two important feeding peptide hormones secreted by gastric endocrine cells, and that pharmacological inhibition of mTOR signaling in these nuclei drives a reduction in food intake. Further, mTOR signaling is an important fuel sensing mechanism which regulates extracellular signals provided by gastric hormones. Together with a previous report by Mori et al (Mori et al., 2009) in which mTOR activation in POMC neurons was demonstrated to block the catabolic function of these neurons, to promote nutrient intake and increase adiposity, these observations suggest a positive relationship between neuronal mTOR activity and food intake in the central nervous system. In contrast, in an earlier study by Cota et al (Cota et al., 2006) using pharmacological approaches to inhibit mTOR signaling in hypothalamus, mTOR activity was shown to be negatively related to energy intake. In addition, activation of mTOR signaling by leucine in the nucleus of the solitary tract has been reported to decrease the meal size (Blouet and Schwartz, 2012). The discrepancy between these experimental results may implicate the presence of differential mechanisms involved in the control of energy homeostasis. The current study supports the concept that the DVC is critical for the control of energy homeostasis based on previous observations that reduction of energy availability in DVC neurons trigger behavioral, endocrine, and autonomic responses which act to restore energy balance, and that NTS neurons express AMPK and contribute to the control food intake (Hayes et al., 2009). Taken together, these studies indicate that mTOR signaling is present and contributes to the control of energy homeostasis across the neuraxis rather than through a localized effect on a single brain region.

Food intake is controlled by the hypothalamus, which integrates a variety of orexigenic and anorexigenic signals from both central and peripheral sources. Ghrelin and nesfatin-1, two important gastric hormones secreted by the X/A like endocrine cells represent hunger and satiety signals have been demonstrated to act on hypothalamic neurons (Nakazato et al., 2001; Oh-I et al., 2006; Stengel et al., 2009; Stengel et al., 2012; Tschop et al., 2000). The current study extends the sites of action for these hormones to the dorsal vagal neurons. Employing a pharmacological approach to alter levels of mTOR activity, we found that both the orexigenic signal ghrelin and the anorexigenic signal nesfatin-1 converge on mTOR signaling in the DMNV neurons to differentially regulate food intake. Unlike the short-term effects of ghrelin and nesfatin-1, ICV injection of rapamycin causes long lasting effects on food intake. mTOR signaling may therefore serve as a potential target for therapeutical strategies targeted at obesity and appetite-related disorders. The upstream signaling moleclues linking the ghrelin and nesfatin-1 with mTOR in the DMNV neurons is currently unknown. Previous studies have reported that AMPK is an important negative regulator for mTOR signaling and that ghrelin may activate AMPK. However, we did not detect an obvious signal for the pAMPK, an active form of AMPK, in the DMNV neurons (unpublished data). The role of AMPK in the mediation of ghrelin’s effect on mTOR signaling in DMNV neurons is unclear.

Limitations exist for both genetic and pharmacological approaches which seek to assess the physiological role of mTOR signaling in control of energy balance. For genetic approaches, chronic changes in levels of mTOR activity may result in altered neuronal circuitry, thereby producing distinct alterations as observed in the study by Mori et al (Mori et al., 2009). Pharmacological approaches are limited by the specifity and the solubility of drugs. However, unlike genetic approaches, alterations induced by pharmacological agents are acute and reversible. It has been reported that mTOR signaling in the hypothalamus can be effectively and reversibly inhibited or activated by 3rd ICV injection of rapamycin and leucine (Cota et al., 2006). As expected, rapamycin and leucine were demonstrated to be effective for the inhibition and stimulation of mTOR signaling respectively when delivered by 4th ICV injection.

In conclusion, this study provides the first evidence that mTOR signaling in the DMNV neurons may integrate both the nutrient and hormonal signals for the maintenance of energy homeostasis. These results imply that mTOR signaling in the hindbrain may serve as a potential target for treatment of obesity and appetite-related disorders.

Acknowledgments

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New Findings

What is the central question of this study?

Does mTOR signaling in the dorsal vagal complex contribute to the modulation of energy homeostasis?

What is the main finding and its importance?

Our study shows for the first time that mTOR signaling in the DMNV neurons regulates both the nutrient and hormonal signals for the modulation of food intake. These results imply that mTOR signaling in the hindbrain may serve as a potential target for treatment of obesity and appetite-related disorders.

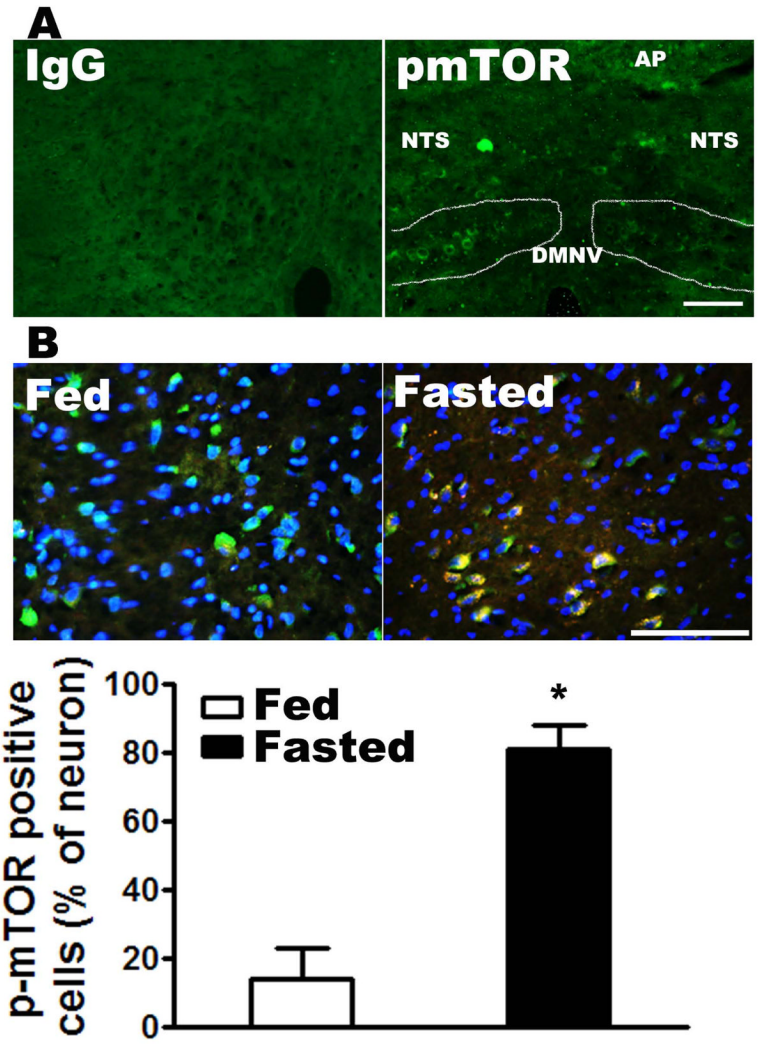

Figure 1. mTOR signaling in DMNV neurons

A. Localization of phosphor-mTOR (Ser2448) in the rat DVC. Left panel, Control antibody showed no staining; Right panel, specific staining of phosphor-mTOR (pmTOR) (green) in DMNV neurons. Nuclei were stained with DAPI (blue). Bar, 20 μm.

B. Effects of fasting on phosphorylation of mTOR in DMNV neurons. Phosphor-mTOR and Hu (a neuronal marker) were stained with TRITC (red) and FITC (green) respectively. Shown are merged stainings (yellow) from fed or 24 h fasted rats. Neurons stained positive for phosphor-mTOR were counted and calculated as percentage of Hu-positive cells. Results are expressed as mean±SEM. * denotes $P<0.05$ relative to fed animals; n=7.

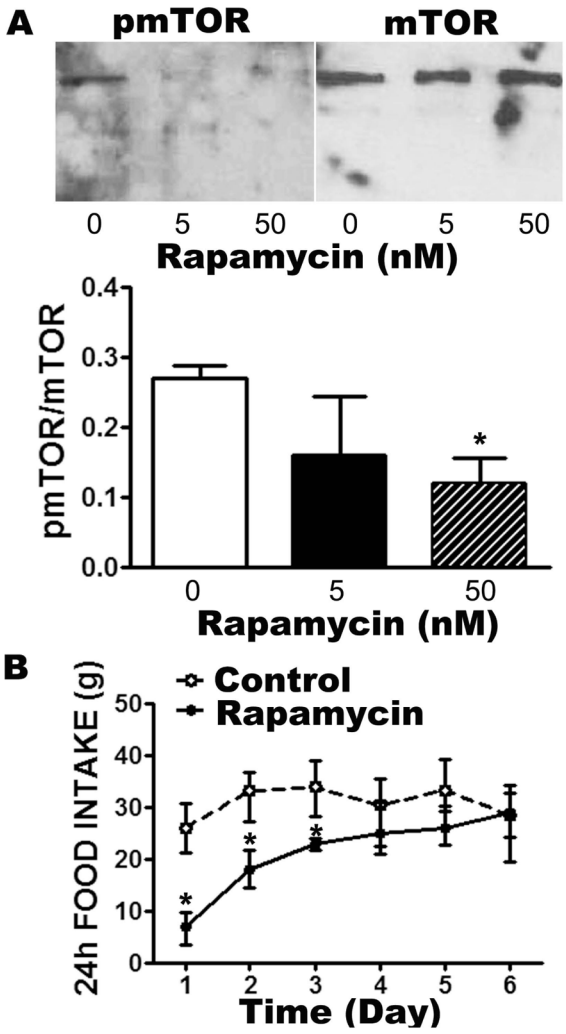

Figure 2. Regulation of food intake by mTOR signaling

A. Inhibition of mTOR signaling by rapamycin. Cultured DMNV neurons were treated with rapamycin at doses of 5 and 50 nM for 30 min. A representative Western blot is shown. Signal intensity of phosphor-mTOR was measured, normalized to total mTOR, and expressed as mean±SEM. * denotes $P<0.05$ relative to control; n=3.

B. Effects of rapamycin on food intake. Rapamycin was administrated by 4th ICV injection. Results are expressed as mean±SEM. * $p<0.05$ relative to control; n=8.

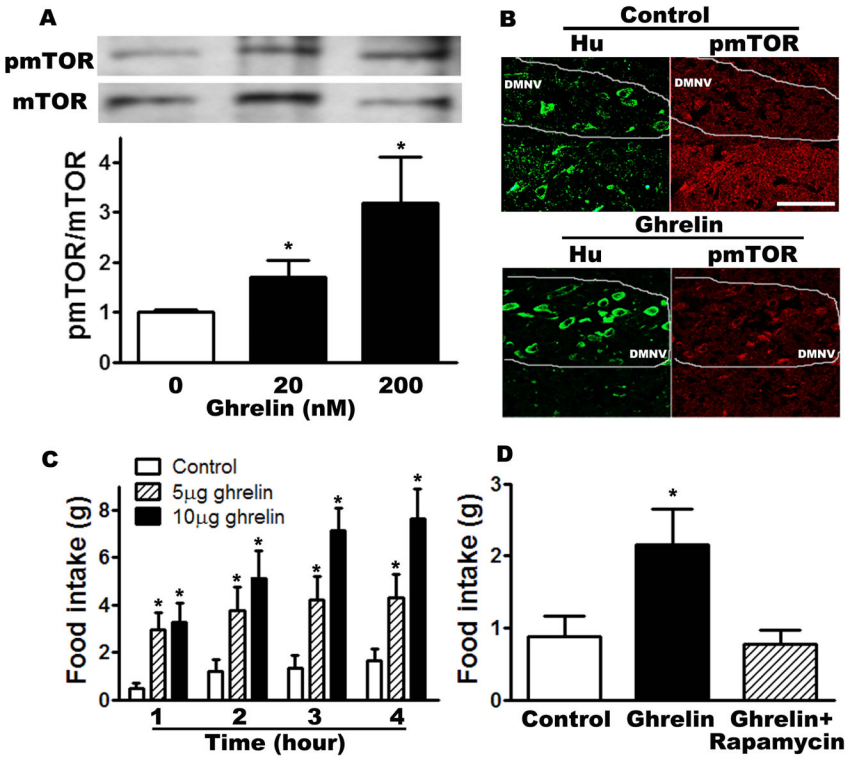

Figure 3. Effects of ghrelin on mTOR signaling and food intake

A. Effects of ghrelin on mTOR signaling in cultured DMNV neurons. Signal intensity of phosphor-mTOR was measured, normalized to total mTOR, and expressed as mean±SEM. * denotes P<0.05 relative to control; n=3.

B. Effects of ghrelin on mTOR signaling in rats. Ghrelin or artificial CSF was administrated by 4th ICV injection. Phosphor-mTOR (red) and Hu (green) were detected by immunofluorsecent staining. Shown are representative resutls from six individual animals.

C. Effects of ghrelin on food intake. Ghrelin administrated by 4th ICV injection induced a significant increase in food intake relative to control during the light phase. Results are expressed as mean±SEM. * denotes P<0.05 relative to control; n=6. There exists a significant difference between the food intake in animals treated with ghrelin at the doses of 5 and 10μg at 3 and 4 hour time points.

D. mTOR-dependent modulation of food intake by ghrelin. Pretreatment with rapamycin for 15 min markedly attenuated the orexigenic effect of ghrelin. Results are expressed as mean ±SEM. * denotes P<0.05 relative to control; n=6.

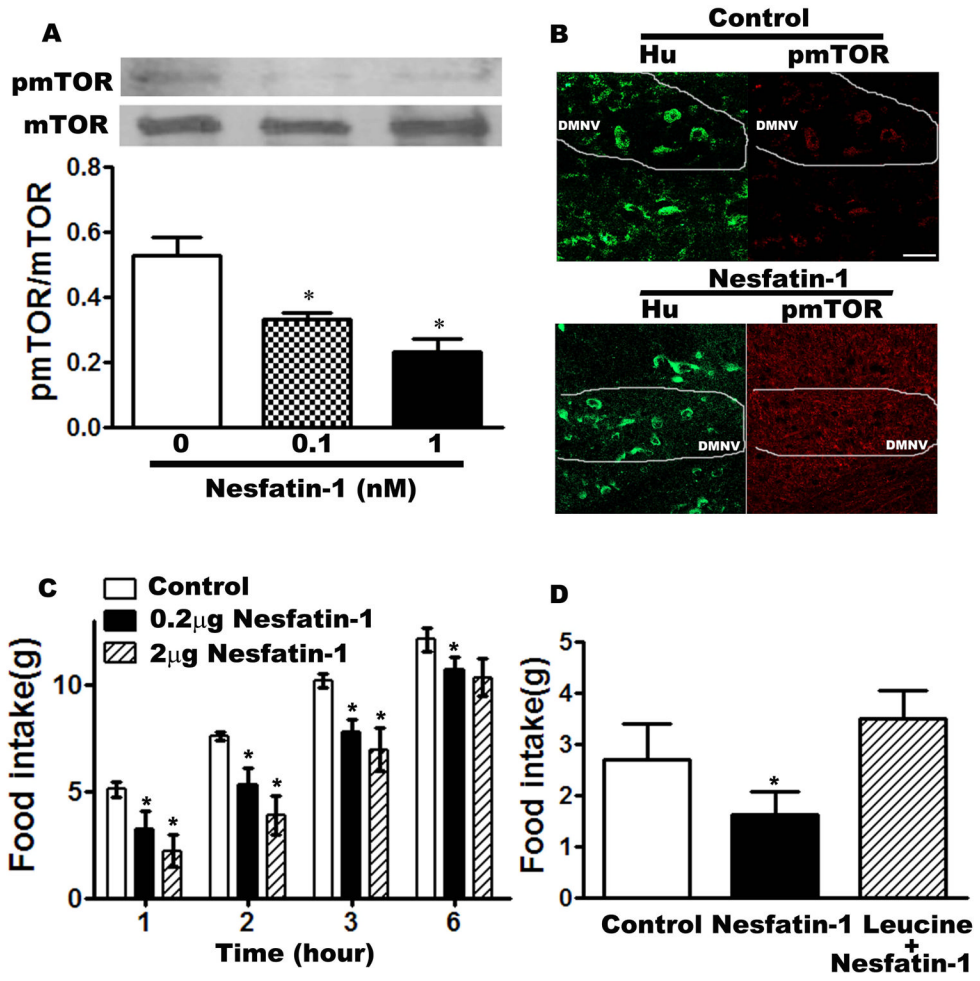

Figure 4. Effects of nesfatin-1 on mTOR signaling and food intake

A. Effects of nesfatin-1 on mTOR signaling in cultured DMNV neurons. Signal intensity of phosphor-mTOR was measured, normalized to total mTOR, and expressed as mean±SEM. * denotes P<0.05 relative to control; n=3.

B. Effects of nesfatin-1 on mTOR signaling in rats. Nesfatin-1 was administrated by 4th ICV injection. Phosphor-mTOR (red) and Hu (green) were detected by immunofluorsecent staining. Shown are representative resutls from six individual animals.

C. Effect of nesfatin-1 on food intake. Nesfatin-1 administrated by 4th ICV injection significantly reduced food intake relative to control. Results are expressed as mean±SEM. * denotes P<0.05 relative to control; n=6.

D. mTOR-dependent modulation of food intake by nesfatin-1. Pretreatment with leucine for 15 min markedly attenuated the anorexigenic effect of nesfatin-1. Results are expressed as mean±SEM. * denotes P<0.05 relative to control; n=6.

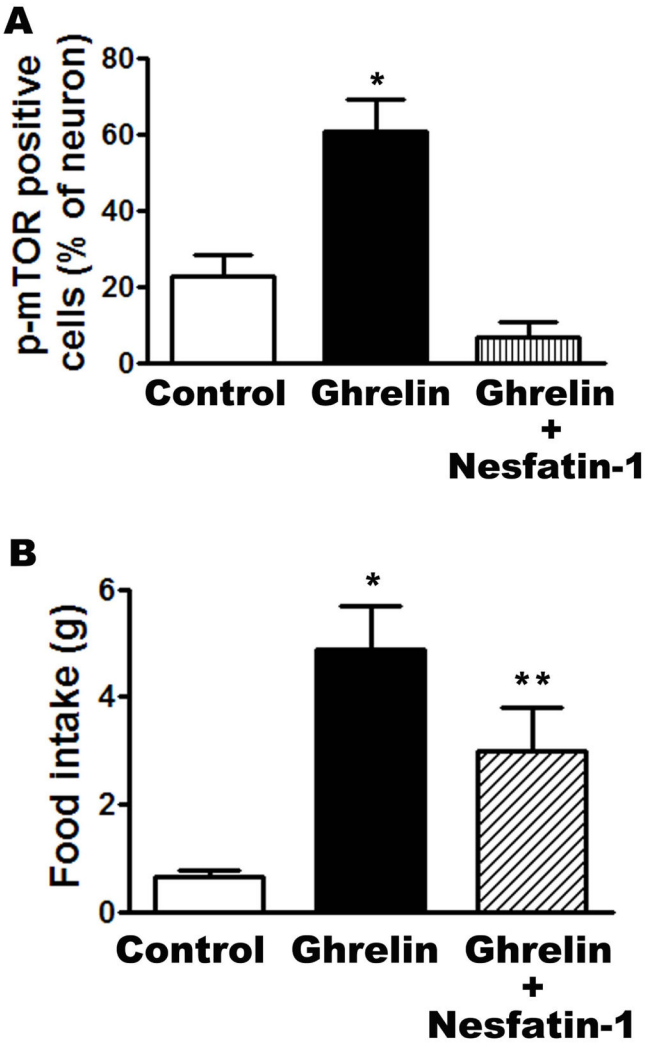

Figure 5. Combined effects of ghrelin and nesfatin-1 on mTOR signalig and food intake

A. Effects on mTOR signaling in vivo. A combination of ghrelin (5 μg) and nesfatin-1 (2 μg), ghrelin (5 μg) alone or vehicle were administrated by 4th ICV injection. Phosphor-mTOR and Hu were detected by immunofluorsecent staining. Neurons stained positively for phosphor-mTOR were counted and expressed as percentage of Hu positive cells. Results were expressed as mean±SEM. * denotes $P<0.05$; n=5.

B. Effects on food intake. Results are expressed as mean±SEM. * denotes $P<0.05$ relative to control; ** denotes $P<0.05$ relative to ghrelin alone; n=5.