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Research Paper

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

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New findings

- **What is the central question of this study?**
  Does mammalian target of rapamycin (mTOR) signalling 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 signalling in neurons of the dorsal motor nucleus of the vagus regulates both the nutrient and the hormonal signals for the modulation of food intake. These results imply that mTOR signalling in the hindbrain may serve as a potential target for treatment of obesity and appetite-related disorders.

Previous studies have demonstrated that mammalian target of rapamycin (mTOR) signalling in the hypothalamus is involved in the control of energy homeostasis. The aim of this study was to characterize the effect of mTOR signalling in the dorsal motor nucleus of the vagus (DMNV) on energy intake. Phospho-mTOR was detected in the DMNV neurons, and its levels were increased by energy deprivation. Rapamycin significantly inhibited mTOR activity and reduced food intake when administrated into the fourth ventricle. Exposure of DMNV neurons to ghrelin increased the phosphorylation of mTOR. Injection of ghrelin into the fourth ventricle significantly increased food intake relative to the control vehicle. Pretreatment with rapamycin for 15 min attenuated the orexigenic effect of ghrelin. A reduction in the phosphorylation of mTOR was observed following injection of nesfatin-1 into the fourth ventricle. When administrated by injection into the fourth ventricle, nesfatin-1 suppressed food intake in comparison with the control vehicle. The anorexigenic effect of nesfatin-1 was significantly attenuated by pretreatment with leucine for 15 min. All these findings suggest that mTOR signalling in the DMNV neurons regulates both the nutrient and the hormonal signals for the modulation of food intake.

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Introduction

The mammalian target of rapamycin (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 signalling (Dennis *et al.* 2001; Inoki *et al.* 2005). Aberrant mTOR activity is linked to diabetes and obesity, and a significant elevation of mTOR signalling has been observed in the 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

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important gastric hormones derived from the X/A-like endocrine cells, ghrelin (Xu *et al.* 2009, 2010) and nesfatin-1 (Li *et al.* 2012), which modulate food intake (Tschop *et al.* 2000; Nakazato *et al.* 2001; Oh-I *et al.* 2006; Stengel *et al.* 2009; Xu *et al.* 2010; Stengel & Taché, 2012) and glucose homeostasis (Sun *et al.* 2006; Yang *et al.* 2010; Xu *et al.* 2012).

Signalling by mTOR in hypothalamic neurons is involved in neuronal sensing of nutrient availability, and regulates food intake and energy balance, suggesting that mTOR signalling in the CNS is crucial for the regulation of energy metabolism (Cota *et al.* 2006; Mori *et al.* 2009). It is currently unknown whether mTOR signalling is present in the dorsal vagal complex (DVC), a group of nuclei critical for the co-ordination of gastrointestinal functions (Zheng *et al.* 2005; Travagli *et al.* 2006; Browning & Travagli, 2011). The DVC, a paired structure located in the dorsal, caudal medulla alongside the central canal, which is contiguous with the fourth ventricle, is composed of three adjacent and functionally integrated nuclei, namely the nucleus tractus solitarii (NTS), the area postrema and the dorsal motor nucleus of the vagus (DMNV). The DVC is a critical relay in vagovagal circuits. Afferent vagal fibres from the gastrointestinal tract synapse on interneurons located within the NTS. These neurons synapse on efferent vagal motor neurons in the DMNV. Preganglionic efferent vagal fibres project to ganglia in the upper gastrointestinal tract, providing parasympathetic control of many aspects of gastrointestinal function, including motility, secretion and absorption (Zheng *et al.* 2005; Travagli *et al.* 2006; Browning & Travagli, 2011).

While fuel-sensing molecules, such as AMP-activated kinase (AMPK; Minokoshi *et al.* 2004; Yang *et al.* 2010; Lam *et al.* 2011) and mTOR (Cota *et al.* 2006; Mori *et al.* 2009), have been implicated in the co-ordination 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 signalling in the dorsal vagal complex. We report that alterations in mTOR signalling 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, USA). β-Fibroblast growth factor (βFGF) was from Invitrogen (Carlsbad, CA, USA). Poly-L-lysine and Triton X-100 were purchased from Sigma-Aldrich (St Louis, MO, USA). Ghrelin and nesfatin-1 peptides were from Phoenix Pharmaceuticals (Burlingame, CA, USA). Rabbit anti-phospho-mTOR (Ser2448) polyclonal antibody, phospho-mTOR (Ser2448) and mTOR mouse monoclonal antibodies, phosphorylated ribosomal protein S6 (pS6; ser235/236) mouse antibody and S6 ribosomal mouse antibody were from Cell Signaling Technology (Beverly, MA, USA). The secondary antibodies fluorescein isothiocyanate-conjugated goat anti-mouse IgG and tetramethylrhodamine isothiocyanate-conjugated goat anti-rabbit IgG were obtained from Jackson ImmunoResearch (West Grove, PA, USA). Intracerebroventricular cannulae were purchased from Plastics One (Roanoke, VA, USA) and cut to 7.4 mm.

Animal care

Animals were housed in a temperature-controlled environment with a 12 h–12 h light–dark cycle and access to food and water *ad libitum* except when indicated.

Cannulation of the fourth ventricle

Male Sprague–Dawley rats weighing 200–250 g were anaesthetized by I.P. injection of a mixture of xylazine and ketamine (13 and 87 mg (kg body weight)$^{-1}$, respectively). A 26 gauge single-lumen cannula was introduced into the fourth ventricle stereotactically according to co-ordinates published by Paxinos and Watson (1998): mid-line, 13 mm caudal to bregma and 7.4 mm deep, with the incisor bar set 3.3 mm below the interaural line. Screws were placed into the skull surrounding the cannula, and the cannula was secured with craniofacial cement. Finally, a sterile internal cannula was placed to maintain patency and prevent infection. On postprocedure day 3, the position of the cannula was tested by intracerebroventricular (I.C.V.) injection of 5-thio-D-glucose (210 μg in 3 μl PBS). A >50% rise in blood glucose in response to 5-thio-D-glucose was considered to be confirmation of the correct position of the cannula. On postprocedure day 7, animals were acclimated and mock-injected with 2 μl of artificial

cerebrospinal fluid. On postprocedure day 9, animals underwent 2 μl I.C.V. injection of rapamycin, leucine, ghrelin, nesfatin-1 or artificial cerebrospinal fluid over 1 min either at 18.00 h in the dark phase or at 08.00 h 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 I.C.V. injection.

Neuronal culture of the dorsal motor nucleus

Dorsal motor nucleus neurons were isolated from neonatal Sprague–Dawley rats (Charles River, Wilmington, MA, USA) as described previously (Zhang *et al.* 2004, 2006). The procedures used for the care and killing of the animals were approved by the University of Michigan Committee on Use and Care of Animals. Briefly, rats were killed by CO_2 inhalation followed by bilateral pneumothorax. The brainstem was rapidly removed and chilled at 0°C in a dissection solution containing (mM): 138 NaCl, 4 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 20 glucose and 10 Hepes. Tissue blocks were prepared and sectioned transversely into 400-μm-thick slices at the level of the obex using a Vibratome 3000 (Redding, CA, USA). The DMNV area was identified under a dissecting microscope as the area immediately ventral to the NTS and dorsal to the XII nucleus. The DMNV tissue was excised and then digested in an enzyme solution containing protease type XIV (0.6 mg ml^{-1}) and trypsin type I (0.4 mg ml^{-1}) at 32°C for 30 min. 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 air supplemented with 5% CO_2 in serum-free culture media consisting of Neurobasal medium containing 2% B27 supplement, 2 mM glutamine, 1% penicillin and streptomycin, and 5 ng ml^{-1} βFGF. After 4 days, one-half of the medium was replaced, and experiments were conducted at 7 days.

Immunofluorescence

Adult Animals were deeply anaesthetized by an I.M. injection of sodium pentobarbital at a dose of 75 mg (kg body weight)$^{-1}$ and killed by bilateral pneumothorax. Intracardiac perfusion with 4% paraformaldehyde, preparation of tissue blocks and sectioning at a thickness of 10 μm were performed as described previously (Zhang *et al.* 2004). Slides at the level of the area postrema were first washed in PBS for 30 min, then incubated with a blocking solution (10% normal goat serum, 3% bovine serum albumin and 0.2% Triton X-100 in PBS, pH 7.4) 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 dilution) and mounted with ProLong Gold antifade reagent. All antibodies were diluted in blocking solution. The primary antibodies used included mouse anti-Hu (1:100 dilution) and rabbit anti-pmTOR (1:100 dilution). The control antibodies used were mouse or rabbit IgG. Tetramethylrhodamine isothiocyanate-conjugated goat anti-rabbit IgG (1:100 dilution) and fluorescein isothiocyanate-conjugated goat anti-mouse IgG (1:100 dilution) were used as secondary antibodies. Slides were viewed using a fluorescence microscope (Nikon Eclipse Ti-U; Nikon Inc., Melville, NY, USA). The DMNV was identified as described in the previous subsection. The positive signal was identified and calculated using the following criteria. Relative optical densities were established for the regions of interest by Image-Pro Plus (Media Cybernetics Inc., Silver Spring, MD, USA). The results were expressed as the difference in grey-scale levels, comparing regions of interest and the background regions in the same section. A change of $\geq$100% 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 phospho-mTOR were counted and expressed as the percentage of Hu-positive cells. Results are given as means ± SEM.

Western blotting

After treatment, DMNV neuronal cultures were homogenized in 60 μl 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 β-glycerophosphate, 1 mM Na_3VO_4, 1 μg ml^{-1} leupeptin and 1 mM phenylmethylsulfonyl fluoride, then centrifuged at 12,000*g* at 4°C for 20 min. Equal amounts of protein (~30–40 μg) from each sample were subjected to electrophoretic separation on a 10% polyacrylamide gel (BioRad, Hercules, CA, USA), then transferred onto an Immobilon™ PVDF membrane (BioRad). Membrane blots were blocked at room temperature for 1 h in 5% milk in Tris-buffered saline (TBS)–Tween 20 (0.05%), then incubated overnight in primary antibodies against mTOR or phospho-mTOR (1:1000 dilution) from Cell Signaling Technology (Danvers, MA, USA). 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, USA) or SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific, Rockford, IL, USA).

Data analysis

Results are expressed as means ± SEM. Data were analysed using ANOVA and Student's unpaired *t* test as appropriate. Significance was accepted as $P < 0.05$.

Results

mTOR signalling in the DVC

As shown in Fig. 1*A*, phospho-mTOR was detected in the majority of DMNV neurons and a few NTS neurons, suggesting the presence of mTOR signalling in the DVC. No positive signal was observed in the control IgG staining. Given that mTOR signalling was detected mainly in the DMNV neurons, we focused our study on this nucleus instead of on the NTS. We first examined alterations in phosphor-mTOR in DMNV neurons in response to fasting. As shown in Fig. 1*B*, 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 *versus* 14 ± 9% in fed animals, $P < 0.01$), suggesting an increase in the mTOR signalling.

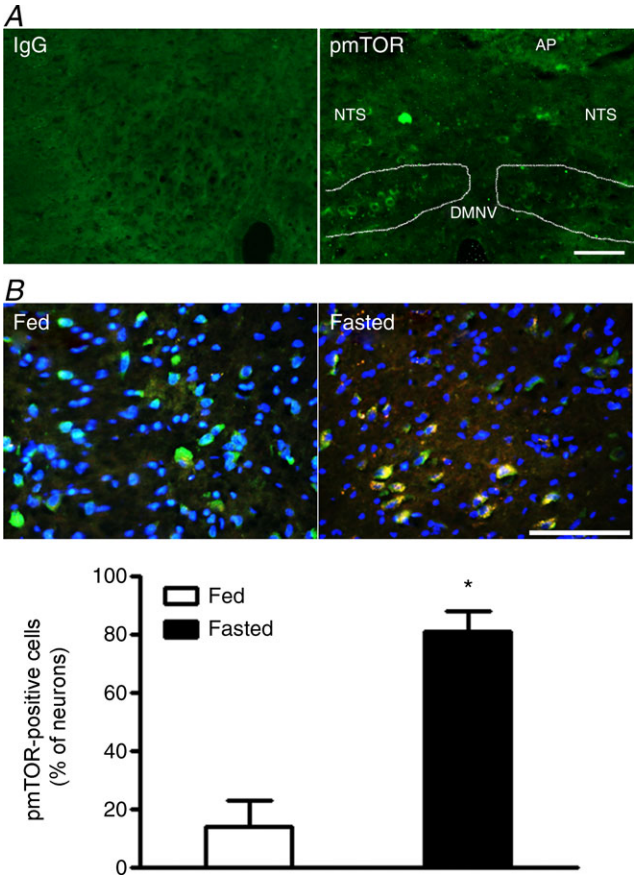

Figure 1. Mammalian target of rapamycin (mTOR) signalling in neurons of the dorsal motor nucleus of the vagus (DMNV)
A, localization of phospho-mTOR (Ser2448) in the rat dorsal vagal complex. Left panel, control antibody showed no staining; and right panel, specific staining (green) of phospho-mTOR (pmTOR) in DMNV neurons. Nuclei were stained with DAPI (blue). Scale bar represents 20 μm. *B*, effects of fasting on phosphorylation of mTOR in DMNV neurons. Phospho-mTOR and Hu (a neuronal marker) were stained with tetramethylrhodamine isothiocyanate (red) and fluorescein isothiocyanate (green), respectively. Shown are merged stainings (yellow) from fed rats or rats fasted for 24 h. Neurons stained positive for phospho-mTOR were counted and calculated as the percentage of Hu-positive cells. Results are shown as means + SEM. $^*P < 0.05$ relative to fed animals; $n = 7$.

Regulation of food intake by mTOR signalling in DMNV neurons

If mTOR signalling contributes to energy homeostasis, one would expect that inhibition of mTOR signalling in the

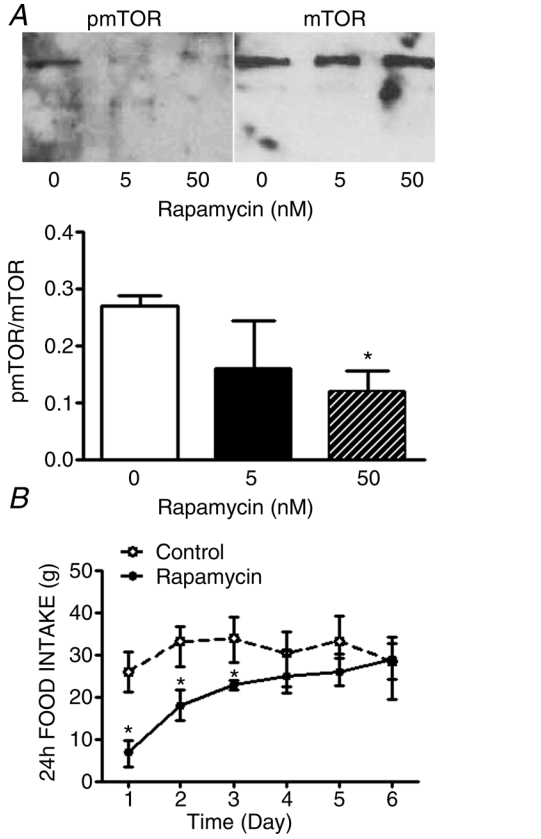

Figure 2. Regulation of food intake by mTOR signalling
A, inhibition of mTOR signalling by rapamycin. Cultured DMNV neurons were treated with rapamycin at doses of 5 and 50 nM for 30 min. A representative Western blot is shown. The signal intensity of phospho-mTOR was measured, normalized to total mTOR and shown as the mean + SEM. $^*P < 0.05$ relative to control; $n = 3$. *B*, effects of rapamycin on food intake. Rapamycin was administrated by fourth intracerebroventricular (I.C.V.) injection. Results are shown as means ± SEM. $^*P < 0.05$ relative to control; $n = 8$.

hindbrain would alter food intake. Rapamycin, a specific inhibitor of mTOR signalling, was used in the following experiments to verify this concept. We first examined the effect of rapamycin on mTOR signalling in primary DMNV cultures. Cells were incubated with different concentrations of rapamycin for 30 min. As shown in Fig. 2*A*, rapamycin at the doses of 5 and 50 nM significantly decreased levels of phospho-mTOR, suggesting inhibition of mTOR signalling in cultured DMNV neurons.

To examine the effect of mTOR signalling in the DVC on food intake, rats were treated with fourth I.C.V. injection of either rapamycin at a dose of 50 μg in 2 μl CSF or vehicle. As shown in Fig. 2*B*, relative to the control rats receiving vehicle administration, animals treated with fourth I.C.V. injection of rapamycin demonstrated a significant decrease in food intake, with persistent effects lasting for up to 3 days.

Effects of ghrelin on mTOR signalling 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 signalling in the DMNV is involved in ghrelin-induced stimulation of food intake. As shown in Fig. 3*A*, treatment of cultured DMNV neurons with 20 and 200 nM of ghrelin for 30 min significantly increased the levels of phosphor-mTOR. The doses of ghrelin were chosen based on the literature and previous experiments in our laboratory. Fourth I.C.V. injection of ghrelin significantly increased the phosphorylation of mTOR in the DMNV neurons stained positively for Hu, a neuronal marker (Fig. 3*B*; 61 ± 14 *versus* $22 \pm 10\%$ of control, $P < 0.05$). Administration of 5 and 10 μg ghrelin into the fourth ventricle significantly increased the food intake during the light phase (Fig. 3*C*). Pretreatment with rapamycin

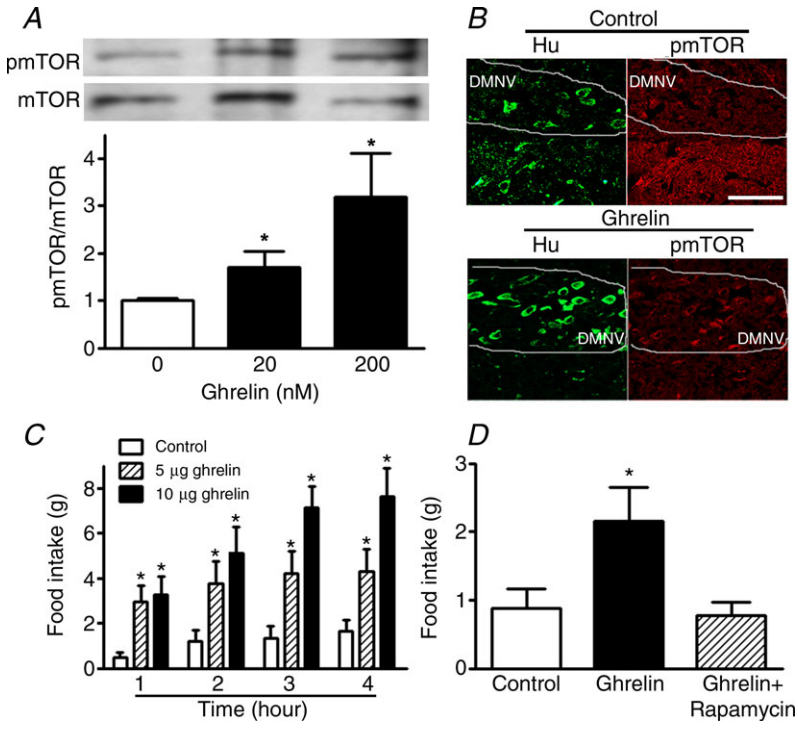

Figure 3. Effects of ghrelin on mTOR signalling and food intake
A, effects of ghrelin on mTOR signalling in cultured DMNV neurons. The signal intensity of phosphor-mTOR was measured, normalized to total mTOR and shown as the mean + SEM. $^*P < 0.05$ relative to control; $n = 3$. *B*, effects of ghrelin on mTOR signalling in rats. Ghrelin or artificial cerebrospinal fluid was administrated by fourth I.C.V. injection. Phospho-mTOR (red) and Hu (green) were detected by immunofluorsecent staining. The results shown are representative of six animals. *C*, effects of ghrelin on food intake. Ghrelin administrated by fourth I.C.V. injection induced a significant increase in food intake relative to control animals during the light phase. Results are shown as means + SEM. $^*P < 0.05$ relative to control; $n = 6$. There is a significant difference between the food intake in animals treated with ghrelin at the doses of 5 and 10 μg at 3 and 4 h 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 shown as means + SEM. $^*P < 0.05$ relative to control; $n = 6$.

(37.5 μg) for 15 min attenuated the orexigenic effect of 10 μg ghrelin (Fig. 3*D*). These results suggest that ghrelin stimulates food intake by activation of mTOR signalling in DMNV neurons.

Effects of nesfatin-1 on mTOR signalling and food intake

Next, the effect of nesfatin-1, a gastric anorexigenic hormone, on mTOR signalling in the DMNV neurons and food intake was investigated. As shown in Fig. 4*A*, cultured DMNV neurons exposed to 0.1 and 1 nM of nesfatin-1 for 30 min demonstrated a significant decrease in levels of phospho-mTOR, suggesting an inhibition of mTOR signalling. A similar reduction in levels of phosphor-mTOR was observed in DMNV neurons when exposed to nesfatin-1 by fourth I.C.V. injection (Fig. 4*B*; (4 ± 5 *versus* 22 ± 10% of control, $P < 0.05$). Nesfatin-1 (0.2 and 2 μg) administrated by fourth I.C.V. injection markedly reduced food intake during the dark phase (Fig. 4*C*). Pretreatment with leucine (1 μg for 15 min), a branched-chain α-amino acid with potent agonism of mTOR signalling, significantly attenuated the anorexigenic effect of 2 μg nesfatin-1 (Fig. 4*D*).

Combined effects of ghrelin and nesfatin-1 on mTOR signalling and food intake

The combined effects of ghrelin and nesfatin-1 were examined. As shown in Fig. 5*A*, simultaneous administration of ghrelin (5 μg) and nesfatin-1 (2 μg) significantly attenuated the activation of mTOR signalling in DMNV neurons relative to ghrelin alone (Fig. 5*A*), accompanied by a marked reduction in food intake relative to ghrelin treatment alone (Fig. 5*B*).

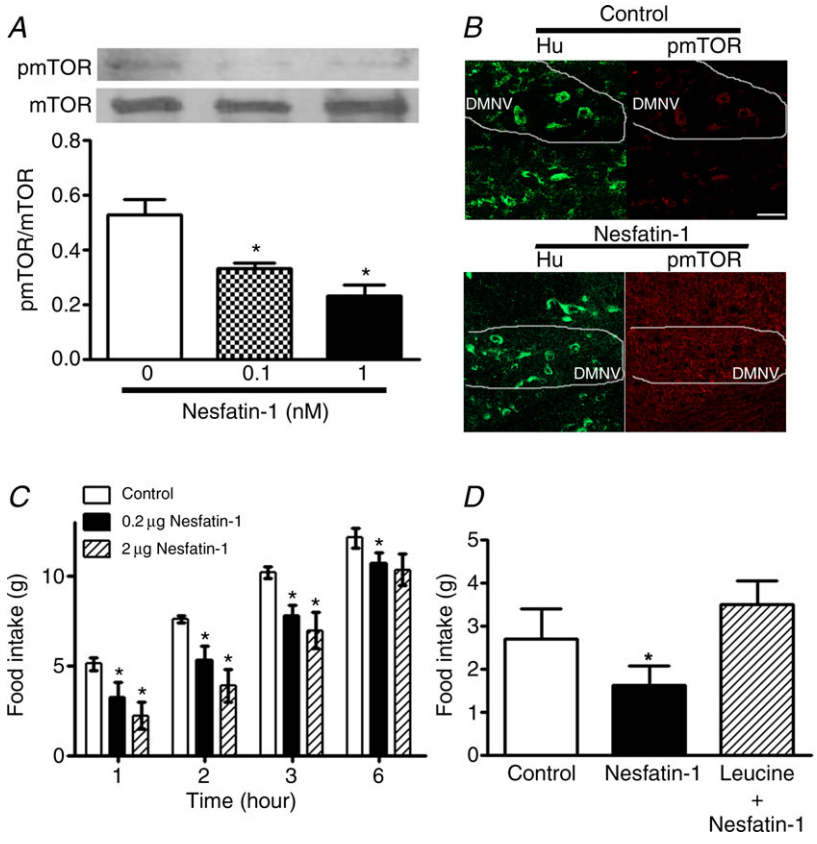

Figure 4. Effects of nesfatin-1 on mTOR signalling and food intake
A, effects of nesfatin-1 on mTOR signalling in cultured DMNV neurons. The signal intensity of phosphor-mTOR was measured, normalized to total mTOR and shown as the mean + SEM. $^*P < 0.05$ relative to control; $n = 3$. *B*, effects of nesfatin-1 on mTOR signalling in rats. Nesfatin-1 was administrated by fourth I.C.V. injection. Phospho-mTOR (red) and Hu (green) were detected by immunofluorsecent staining. The results shown are representative of six animals. *C*, effect of nesfatin-1 on food intake. Nesfatin-1 administrated by fourth I.C.V. injection significantly reduced food intake relative to control animals. Results are shown as means + SEM. $^*P < 0.05$ 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 shown as means + SEM. $^*P < 0.05$ relative to control; $n = 6$.

Discussion

The present study demonstrates that mTOR signalling in DMNV neurons is involved in the regulation of food intake. This general conclusion is supported by the following five distinct observations: (i) mTOR signalling is present in DMNV neurons, and its activity is upregulated by fasting; (ii) inhibition of mTOR signalling by rapamycin decreases food intake; (iii) ghrelin administrated by fourth I.C.V. injection markedly increases food intake, and this effect is mediated by activation of mTOR signalling in DMNV neurons; (iv) in contrast, nesfatin-1 decreases food intake through inhibition of mTOR signalling in DMNV neurons; and (v) simultaneous administration of ghrelin and nesfatin-1 blunts increases in mTOR signalling and food intake induced by ghrelin.

Signalling by mTOR 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).

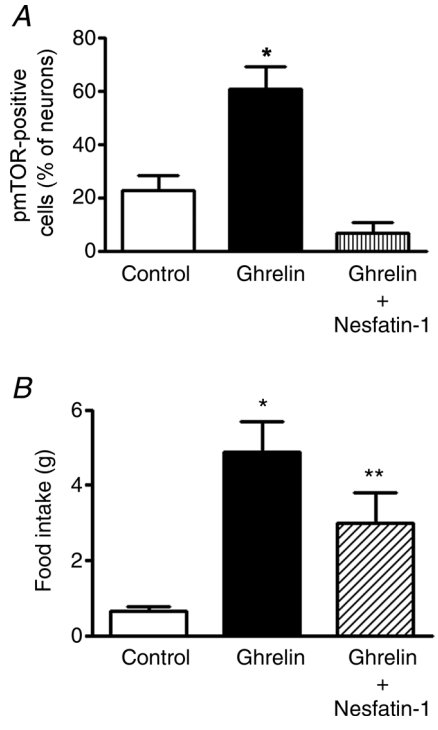

Figure 5. Combined effects of ghrelin and nesfatin-1 on mTOR signalling and food intake
A, effects on mTOR signalling *in vivo*. A combination of ghrelin (5 μg) and nesfatin-1 (2 μg), ghrelin (5 μg) alone or vehicle was administrated by fourth I.C.V. injection. Phospho-mTOR and Hu were detected by immunofluorsecent staining. Neurons stained positively for phospho-mTOR were counted and expressed as a percentage of Hu-positive cells. Results are shown as means + SEM. $^*P < 0.05$; $n = 5$. *B*, effects on food intake. Results are shown as means + SEM. $^*P < 0.05$ relative to control; $\dagger P < 0.05$ relative to ghrelin alone; $n = 5$.

Whether extrahypothalamic mTOR signalling in the CNS contributes to the control of energy homeostasis has not been previously demonstrated. Here, we demonstrate that mTOR signalling 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 signalling in these nuclei drives a reduction in food intake. Furthermore, mTOR signalling is an important fuel-sensing mechanism, which regulates extracellular signals provided by gastric hormones. Together with a previous report by Mori *et al.* (2009), in which mTOR activation in proopiomelanocortin (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 CNS. In contrast, in an earlier study by Cota *et al.* (2006), using pharmacological approaches to inhibit mTOR signalling in hypothalamus, mTOR activity was shown to be related negatively to energy intake. In addition, activation of mTOR signalling by leucine in the NTS has been reported to decrease the meal size (Blouet & Schwartz, 2012). The discrepancy between these experimental results may implicate the presence of differential mechanisms involved in the control of energy homeostasis. The present study supports the concept that the DVC is critical for the control of energy homeostasis based on previous observations that a reduction of energy availability in DVC neurons triggers behavioural, endocrine and autonomic responses that act to restore energy balance, and that NTS neurons express AMPK and contribute to the control of food intake (Hayes *et al.* 2009). Taken together, these studies indicate that mTOR signalling 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 that have been demonstrated to act on hypothalamic neurons (Tschop *et al.* 2000; Nakazato *et al.* 2001; Oh-I *et al.* 2006; Stengel *et al.* 2009; Stengel & Taché, 2012). The present 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 signalling in the DMNV neurons to regulate food intake differentially. Unlike the short-term effects of ghrelin and nesfatin-1, I.C.V. injection of rapamycin causes long-lasting effects on food intake. Signalling by mTOR may therefore serve as a potential target for

therapeutical strategies targeted at obesity and appetite-related disorders. The upstream signalling molecules linking ghrelin and nesfatin-1 with mTOR in the DMNV neurons are currently unknown. Previous studies have reported that AMPK is an important negative regulator for mTOR signalling and that ghrelin may activate AMPK; however, we did not detect an obvious signal for pAMPK, an active form of AMPK, in the DMNV neurons (Zhang W, Zhang C, MW Mulholland, unpublished data). The role of AMPK in mediation of the effect of ghrelin on mTOR signalling in DMNV neurons is unclear.

Limitations exist for both genetic and pharmacological approaches that seek to assess the physiological role of mTOR signalling in the control of energy balance. For genetic approaches, chronic changes in the levels of mTOR activity may result in altered neuronal circuitry, thereby producing distinct alterations, as observed in the study by 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 signalling in the hypothalamus can be effectively and reversibly inhibited or activated by third I.C.V. injection of rapamycin or leucine, respectively (Cota *et al.* 2006). As expected, rapamycin and leucine were demonstrated to be effective for the inhibition and stimulation of mTOR signalling, respectively, when delivered by fourth I.C.V. injection.

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

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Additional information

Competing interests

None declared.

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