Cell Metabolism
Article

Hypothalamic Fatty Acid Metabolism Mediates the Orexigenic Action of Ghrelin

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DOI 10.1016/j.cmet.2008.03.006

SUMMARY

Current evidence suggests that hypothalamic fatty acid metabolism may play a role in regulating food intake; however, confirmation that it is a physiologically relevant regulatory system of feeding is still incomplete. Here, we use pharmacological and genetic approaches to demonstrate that the physiological orexigenic response to ghrelin involves specific inhibition of fatty acid biosynthesis induced by AMP-activated protein kinase (AMPK) resulting in decreased hypothalamic levels of malonyl-CoA and increased carnitine palmitoyltransferase 1 (CPT1) activity. In addition, we also demonstrate that fasting downregulates fatty acid synthase (FAS) in a region-specific manner and that this effect is mediated by an AMPK and ghrelin-dependent mechanisms. Thus, decreasing AMPK activity in the ventromedial nucleus of the hypothalamus (VMH) is sufficient to inhibit ghrelin's effects on FAS expression and feeding. Overall, our results indicate that modulation of hypothalamic fatty acid metabolism specifically in the VMH in response to ghrelin is a physiological mechanism that controls feeding.

INTRODUCTION

Feeding requires the integration within the central nervous system (CNS) of multiple afferent energy-related homeostatic signals that interact with specific hypothalamic nuclei ordered into complex neuronal circuits. These nuclei respond to changes in energy status by altering the expression of specific neuropeptides and by inducing structural changes to their neuronal networks in order to adjust food intake to whole-body energy demands (Flier, 2004; Pinto et al., 2004; Pagotto et al., 2006; Plum et al., 2006; Morton et al., 2006). It is unclear how the expression of these neuropeptides is regulated and by which specific signals and through what efferent systems they accurately match the bioenergetic needs of the organism. Recent evidence suggests that nutrient-related metabolic pathways, such as fatty acid metabolism, may act as direct modulators of the hypothalamic control of feeding (Loftus et al., 2000; Obici et al., 2003; He et al., 2006; Pocai et al., 2006).

The de novo fatty acid biosynthesis pathway comprises three key enzymes, acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), and malonyl-CoA decarboxylase (MCD), all of which are expressed in the hypothalamus (Kahn et al., 2005; López et al., 2006). Malonyl-CoA is not only an intermediary in the biosynthesis of fatty acids but also plays an important role in the metabolic switch that controls the balance between de novo lipogenesis and fatty acid oxidation. Levels of malonyl-CoA depend on the balance between ACC activity that catalyzes malonyl-CoA synthesis and the activation of FAS and MCD that facilitates its clearance. AMP-activated protein kinase (AMPK) is an important regulator of the fatty acid biosynthetic pathway. Following activation, AMPK phosphorylates and inhibits ACC, preventing the production of malonyl-CoA. This further stimulates carnitine palmitoyltransferase 1 (CPT1), ultimately facilitating mitochondrial fatty acid oxidation (Obici et al., 2003; Kahn et al., 2005; Wolfgang et al., 2006). Pharmacologic and genetic evidence has demonstrated that altered levels and activity of these enzymes modulate feeding (Loftus et al., 2000; Obici et al., 2003; Minokoshi et al., 2004; Wolfgang et al., 2006; López et al., 2006; Chakravarthy et al., 2007). Since these manipulations altered the hypothalamic pool of malonyl-CoA (Hu et al., 2003; López

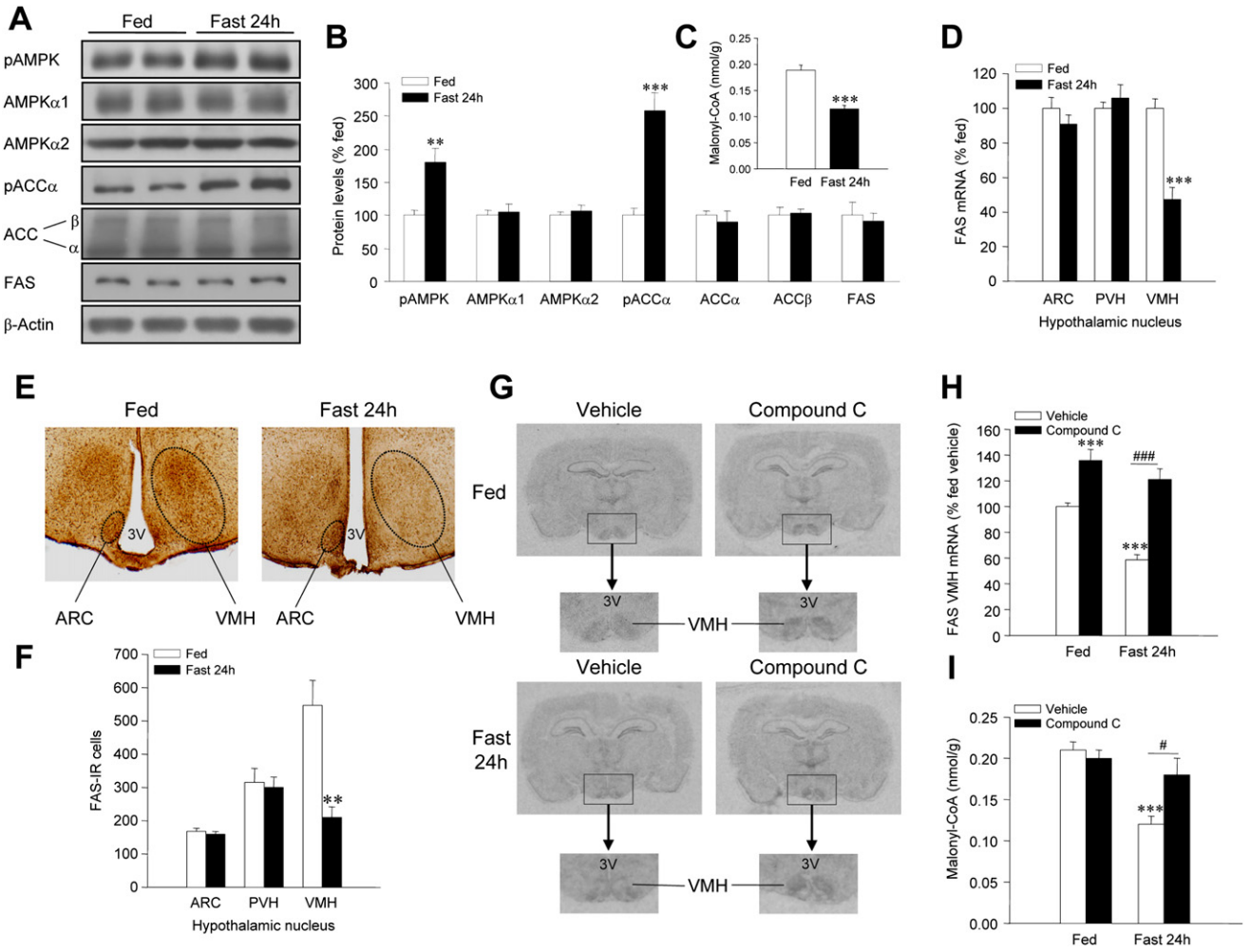

Figure 1. Fasting Influences Hypothalamic Fatty Acid Metabolism

(A) Western blot; (B) hypothalamic protein levels (±SEM) of pAMPK, AMPKα1, AMPKα2, pACCα, ACCα, ACCβ, and FAS; and (C) hypothalamic malonyl-CoA levels (±SEM) in fed and 24 hr fasted rats. (D) FAS mRNA levels (±SEM) in the ARC, PVH, and VMH of fed and 24 hr fasted rats. (E) Hypothalamic FAS-IR (40×) in fed and 24 hr fasted rats. (F) Measurement of FAS-IR cells (±SEM) in the ARC, PVH, and VMH of fed and 24 hr fasted rats. (G) Expression of FAS in the VMH (upper: 5×; lower: 20×), (H) FAS mRNA levels (±SEM) in the VMH, and (I) hypothalamic malonyl-CoA levels (±SEM) of the described groups are shown. 3V: third ventricle; $^{**}p < 0.01$ versus fed; $^{***}p < 0.001$ versus fed or fed/vehicle; $^{\#}p < 0.05$ fast 24 hr/vehicle versus fast 24 hr/compound C; $^{\#\#\#}p < 0.001$ fast 24 hr/vehicle versus fast 24 hr/compound C.

et al., 2006; Chakravarthy et al., 2007) and/or long-chain fatty acids-CoA (LCFA-CoA) (Obici et al., 2003; He et al., 2006; Pocai et al., 2006), these metabolites have been proposed as signals of nutrient abundance able to modulate feeding.

Ghrelin (a hormone produced in the stomach with orexigenic properties) has recently attracted enormous interest as a potential antiobesity therapeutic target (Foster-Schubert and Cummings, 2006). Chronic ghrelin administration promotes weight gain and adiposity (Tschop et al., 2000). Also, obese patients diagnosed with Prader-Willi syndrome have increased levels of ghrelin (Cummings et al., 2002). Furthermore, loss-of-function experiments using ghrelin knockout (KO) mice (Wortley et al., 2005) or ghrelin receptor KO (GHS-R KO) mice (Zigman et al., 2005) have shown that lack of ghrelin function protects against early-onset obesity. Altogether, these studies indicate that ghrelin might be an important signal for humans and rodents to prepare for meal initiation (Cummings et al., 2001; Drazen et al., 2006).

In this study, we show that increased ghrelin levels, in the context of food deprivation, promote feeding through AMPK-mediated modulation of hypothalamic fatty acid metabolism, thus leading to decreased malonyl-CoA and increased CPT1 activity. Altogether these data demonstrate a physiological role for hypothalamic fatty acid metabolism modulation as the mechanism activated by ghrelin.

RESULTS

Fasting Induces Phosphorylation of Hypothalamic AMPK and Decreases FAS in the VMH

Fasting stimulated the phosphorylation of hypothalamic AMPK and ACCα (Figures 1A and 1B), resulting in a decrease in

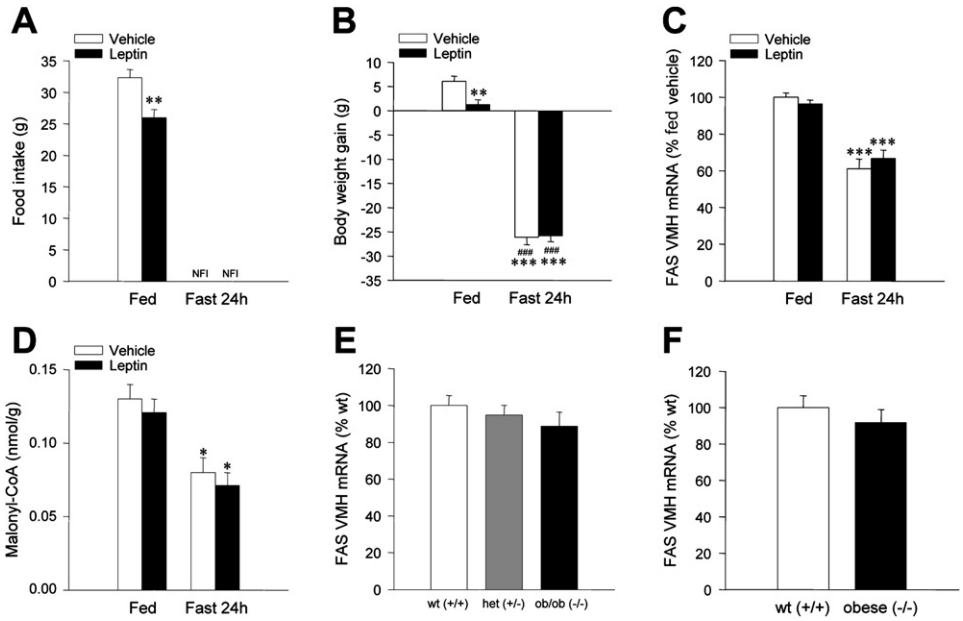

Figure 2. Leptin Fails to Regulate Hypothalamic Fatty Acid Metabolism

(A) Food intake (±SEM) and (B) body weight change (±SEM) of rats treated with IP injections of vehicle or leptin are shown. (C) FAS mRNA levels (±SEM) in the VMH and (D) hypothalamic malonyl-CoA levels (±SEM) of the described groups are also shown. FAS mRNA levels (±SEM) in the VMH of ob/ob mice (E) and obese Zucker rats (F) are shown together with lean controls. NFI: non-food intake; *p < 0.05 versus fed/vehicle; **p < 0.01 versus fed/vehicle; ***p < 0.001 versus fed/vehicle; ###p < 0.001 versus fed/leptin.

hypothalamic malonyl-CoA levels (Figure 1C). FAS mRNA (Figures S1A and S1B available online) and protein (Figures S1C–S1E) were specifically detected in the arcuate (ARC), paraventricular (PVH), and ventromedial (VMH) nuclei, where FAS was coexpressed with brain-derived neurotrophic factor (BDNF) (Figure S1E). Total levels of FAS protein in the hypothalamus did not change after 24 hr fasting (Figures 1A and 1B). However, 24 hr fasting selectively decreased FAS mRNA expression (Figure 1D) and FAS-immunoreactive cells in the VMH (Figures 1E and 1F).

AMPK Signaling Regulates Fasting-Induced Inactivation of Hypothalamic ACC and FAS in the VMH

To establish a link between fasting, phosphorylation of AMPK, and inhibition of ACC and FAS, we investigated the effect of compound C, a nonspecific inhibitor of AMPK (Zhou et al., 2001; Bain et al., 2007), in fasted rats. Compound C clearly inhibited the AMPK pathway as demonstrated by the decreased phosphorylation of AMPK and ACCα (Figures S2A and S2B). Interestingly, compound C also caused a reduction in the expression of Ca^{2+}/calmodulin-dependent protein kinase kinase (CaMKKα), which has previously been reported to phosphorylate AMPK in vitro (Hawley et al., 2005; Woods et al., 2005) (Figures S2A and S2B). In contrast, compound C had no significant effect on a number of other protein kinase pathways studied (Figures S2A and S2B), including some of those that have recently been reported to be inhibited by high doses of compound C in vitro (Bain et al., 2007).

Intracerebroventricular (ICV) administration of compound C to 24 hr fasted rats prevented the previously observed fasting-induced physiological decrease of FAS mRNA levels in the VMH (Figures 1G and 1H). This suggests that the effect of fasting in decreasing FAS mRNA expression in the VMH involves an AMPK-dependent mechanism. AMPK inhibition by compound C also resulted in activation of ACC. As a result of this, malonyl-CoA levels remained elevated at fed-state levels despite rats being fasted for 24 hr (Figure 1I).

Decreased Leptin and Leptin Signaling Are Not Involved in Fasting-Induced Changes of ACC and FAS in the VMH

We initially hypothesized that fasting-induced hypoleptinemia (Table S1) may mediate a fasting-induced decrease of ACC activity and FAS expression in hypothalamus. Fed rats treated with leptin showed a marked decrease in both food intake and body weight (Figures 2A and 2B). Both FAS mRNA levels in the VMH and hypothalamic malonyl-CoA decreased by fasting, and their levels were not restored by leptin administration (Figures 2C and 2D). No changes in FAS mRNA levels in the VMH of fed *ob/ob* mice and fed obese Zucker rats were detected when compared to their respective controls (Figures 2E and 2F). These results show that FAS expression in the VMH and hypothalamic ACC activity are not regulated by leptin, either in the fed or fasted state.

The Orexigenic Effect of Ghrelin Is Mediated by Hypothalamic AMPK

AMPK has recently been proposed as a target of ghrelin in the hypothalamus (Andersson et al., 2004; Kohno et al., 2008). However, this hypothesis has not been confirmed by data linking AMPK stimulation with ghrelin orexigenic activity. ICV administration of ghrelin to fed satiated rats induced a marked increase in their food intake (Figures 3A and S3A). Under these

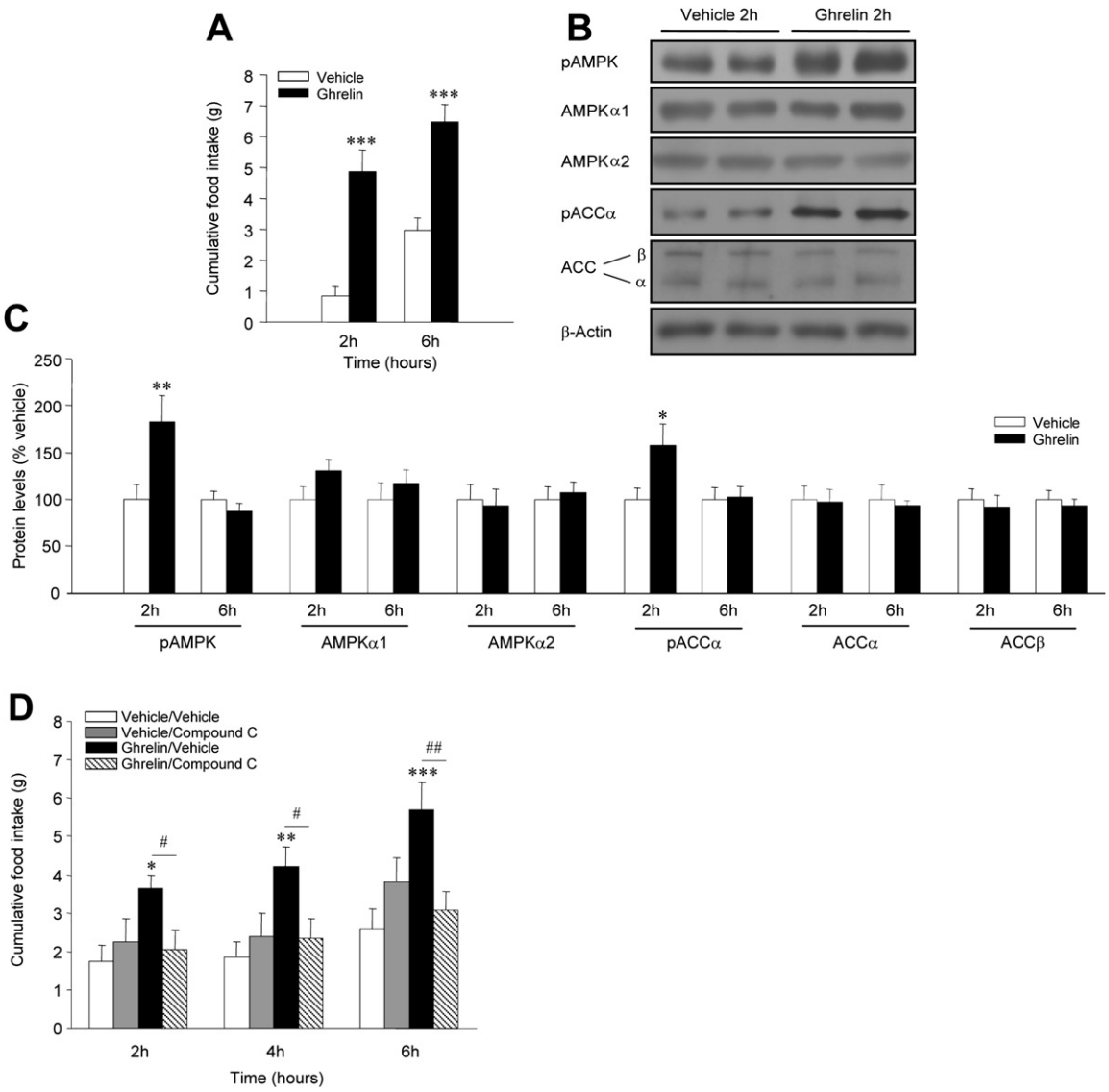

Figure 3. Ghrelin Activates Hypothalamic AMPK and ACC

(A) Cumulative food intake (±SEM), (B) western blot, and (C) hypothalamic protein levels (±SEM) of pAMPK, AMPKα1, AMPKα2, pACCα, ACCα, and ACCβ in vehicle and ghrelin ICV-treated rats are shown. (D) shows cumulative food intake (±SEM) after ICV administration of vehicle or compound C prior to ICV administration of vehicle or ghrelin. *$p < 0.05$ versus vehicle or vehicle/vehicle; **$p < 0.01$ versus vehicle or vehicle/vehicle; ***$p < 0.001$ versus vehicle or vehicle/vehicle; #$p < 0.05$ ghrelin/vehicle versus ghrelin/compound C; ##$p < 0.01$ ghrelin/vehicle versus ghrelin/compound C.

conditions ghrelin administration induced a time-dependent increase in AMPK and ACCα phosphorylation levels, peaking acutely at 1 (data not shown) and 2 hr and returning to normal levels by 6 hr post-treatment (Figures 3B and 3C). To determine whether the orexigenic effect of ghrelin was AMPK dependent we used compound C. Our data demonstrated that ICV administration of compound C to fed rats completely prevented the central orexigenic effect of ghrelin (Figure 3D). The same dose of compound C when administered alone did not have any effect on feeding, excluding the possibility of a direct anorectic effect.

Compound C has been shown to inhibit a number of protein kinases in vitro, suggesting its lack of specificity on AMPK (Bain et al., 2007); thus in order to confirm the link between AMPK and ghrelin orexigenic effects, we adopted a molecular approach to inhibit AMPK, using dominant-negative forms of AMPKα1 and α2 (α1α2 DN). Adenoviruses harboring α1α2 DN (Woods et al., 2000) were injected stereotaxically into the VMH (Figure 4A). Infection efficiency was demonstrated by hypothalamic expression of GFP expressed by the adenovirus construct (Figure 4B) specifically in the VMH (Figure 4C) and also by the fact that pACC levels were significantly decreased in the hypothalamus of ghrelin-treated rats injected with α1α2 DN when compared to ghrelin-treated rats injected with control adenovirus containing GFP (Ghrelin/GFP: 100 ± 10.6; Ghrelin/α1α2 DN: 63.5 ± 6.5; $p < 0.01$). Our data demonstrate that stereotaxic delivery of α1α2 DN in the VMH robustly impaired the central orexigenic effect of ghrelin (Figure 4D).

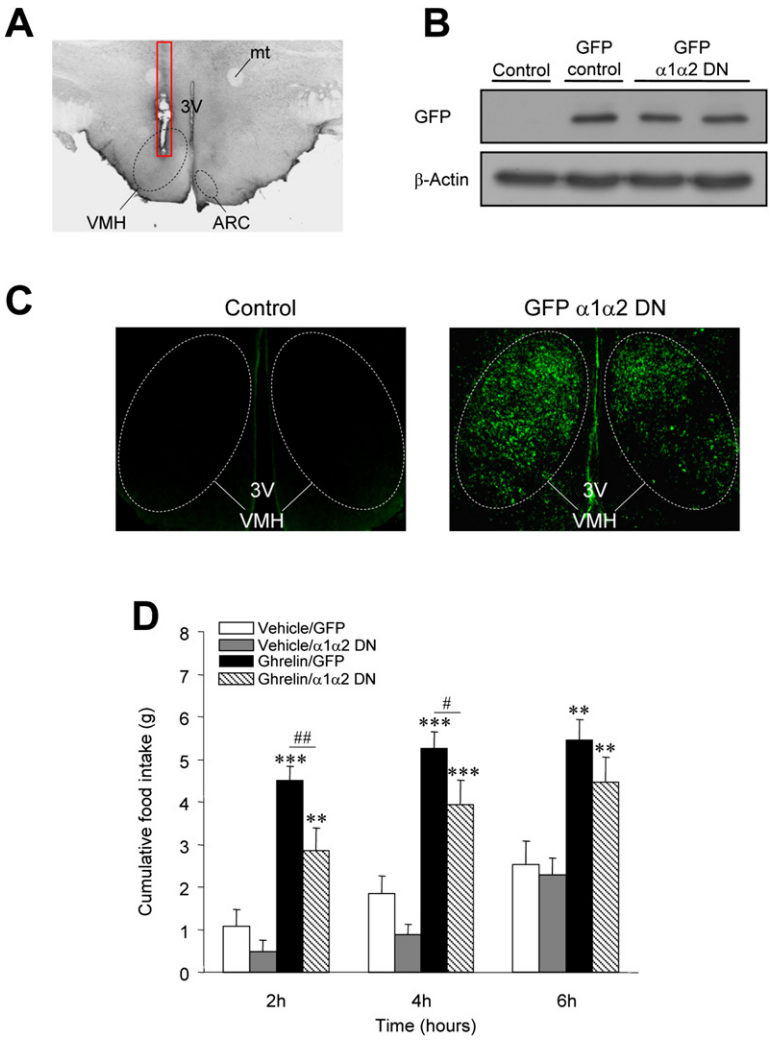

Figure 4. The Orexigenic Effect of Ghrelin Is Mediated by AMPK in the VMH

(A) Coronal section of a rat brain at the level of the hypothalamus showing the injection route for a unilateral stereotaxic microinjection of adenoviral expression vectors. The injection route, enclosed in a red rectangle, is precisely placed in the VMH.

(B) Western blot showing GFP protein levels in the hypothalamus of sham control rats (non-adenovirus-treated, negative control), rats treated with a GFP-expressing adenovirus (positive control), and rats treated with α1α2 DN adenovirus in the VMH.

(C) Representative immunofluorescence with anti-GFP antibody showing GFP expression in the VMH of sham control rats (non-adenovirus-treated, negative control) and rats treated with α1α2 DN adenovirus in the VMH; as expected nonpositive signal was detected in the VMH of sham control rats.

(D) Cumulative food intake (±SEM) after ICV administration of vehicle or ghrelin to rats stereotaxically treated with a GFP-expressing adenovirus or α1α2 DN adenovirus. mt: mammillothalamic tract; 3V: third ventricle; **p < 0.01 versus vehicle/GFP; ***p < 0.001 versus vehicle/GFP; #p < 0.05 ghrelin/GFP versus ghrelin/α1α2 DN; ##p < 0.01 ghrelin/GFP versus ghrelin/α1α2 DN.

Central Administration of Ghrelin Decreases FAS Expression in the Hypothalamus

Central (ICV) ghrelin administration induced a time-dependent specific increase in food intake (Figures 3A and S3A) and a time-dependent decrease in FAS mRNA levels in the VMH in fed rats (Figures 5A, 5B, and S3C), as well as a decrease in total hypothalamic FAS activity (Figure 5C) and protein levels (Figures 5D and 5E) after 6 hr of treatment. No changes in FAS mRNA levels were found either in other hypothalamic nuclei, such as ARC and PVH, or in other brain areas, such amygdala (Amyg), striatum, habenula (Hb), fields CA1, CA2, and CA3 of the hippocampus (HippCA1, 2, 3), hippocampus dentate gyrus (HippDG), motor cortex, pyriform cortex, sensory cortex, substantia nigra (SN), and zona incerta (thalamus, ZI), at any of the evaluated doses (Figure S3C). The decrease in FAS expression was associated with reduced levels of sterol regulatory element binding protein-1 (SREBP1) (Figures 5D and 5E). Peripheral (intraperitoneal, IP) administration of ghrelin induced a marked orexigenic response (Figure S3B) and reproduced the specific inhibitory effect on FAS mRNA expression in the VMH (Figures 5F and S3D). Overall, these results suggest that peripheral ghrelin regulates hypothalamic FAS.

Since agouti-related protein (AgRP) and neuropeptide Y (NPY) expression increases after central ghrelin administration (Seoane et al., 2003; Smith, 2005) (Figure S3E), we initially hypothesized that the effects of ghrelin on FAS may be mediated by the upregulation of these neuropeptides. However, our results showed that ICV administration of AgRP or NPY (Figure S3F) did not affect FAS mRNA. The specificity of the ghrelin effect on FAS in the VMH is further supported by the fact that double immunocytochemistry of FAS and GHS-R revealed that 86.1% ± 1.4% immunoreactive cells in the VMH expressing FAS also coexpressed GHS-R (Figure 5G), whereas other hypothalamic nuclei did not display such a level of coexpression (ARC: 57.6% ± 2.4% of double-stained cells; $p < 0.001$ versus VMH; PVH: 41.0% ± 2.5% of double-stained cells; $p < 0.001$ versus VMH). Overall the data suggest that ghrelin may exert a direct action on FAS-expressing neurons in the VMH.

Ghrelin Mediates the Fasting-Induced Decrease of FAS in the VMH

We hypothesized that the fasting-induced decrease in FAS expression could be mediated by an elevated ghrelin tone (Table S1) on FAS neurons. We first evaluated the effect on FAS mRNA of a ghrelin antagonist, BIM-28163 (Halem et al., 2005), when administered centrally in 24 hr fasted rats. Our data showed that pharmacological blockage of ghrelin action attenuated the fasting-induced decrease of FAS mRNA (Figures 5H and 5J). Second, we examined the effects of fasting on FAS expression in GHS-R KO characterized by a complete lack of ghrelin signaling (Smith, 2005; Zigman et al., 2005). In response to fasting, GHS-R KO mice did not show decreased FAS mRNA levels in the VMH (Figures 5I and 5K). Therefore, our results are consistent with the hypothesis that endogenous ghrelin mediates the effect of fasting on FAS expression.

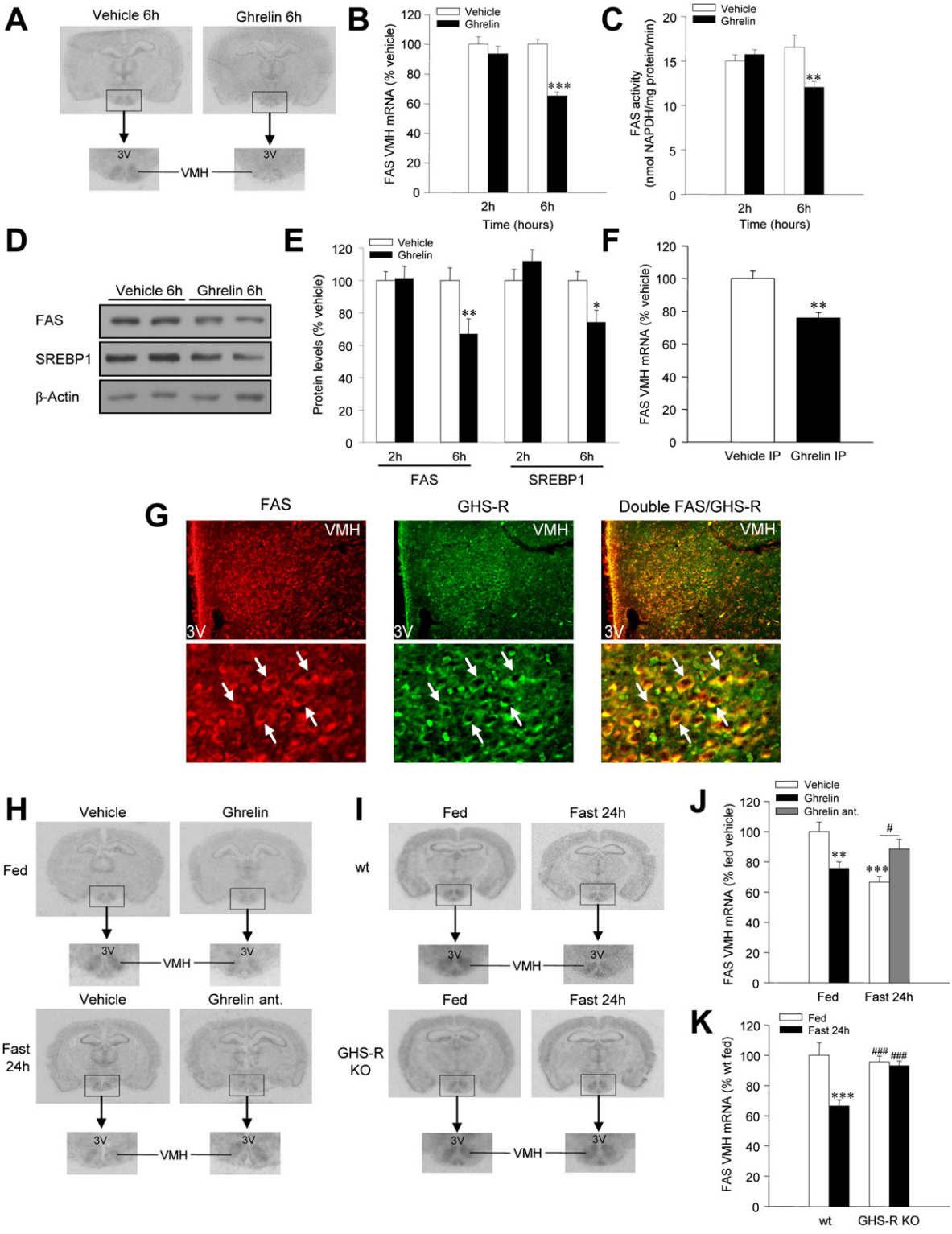

Figure 5. Ghrelin Inhibits FAS Expression Specifically in the VMH

(A) Expression of FAS in the VMH and (B) FAS mRNA levels (±SEM) of vehicle and ghrelin ICV-treated rats (upper: 5×; lower: 20×) are shown. (C) Hypothalamic FAS activity (±SEM), (D) western blot, and (E) hypothalamic FAS and SREBP1 protein levels (±SEM) of vehicle and ghrelin ICV-treated rats are shown for 2 and 6 hr. (F) shows FAS mRNA levels (±SEM) of vehicle and ghrelin IP-treated rats. (G) Double immunohistochemistry (upper: 40×; lower: 100×) shows FAS and GHS-R coexpression in the VMH. (H and I) Expression of FAS in the VMH (upper: 5×; lower: 20×) and (J and K) FAS mRNA levels (±SEM) in the VMH of the described

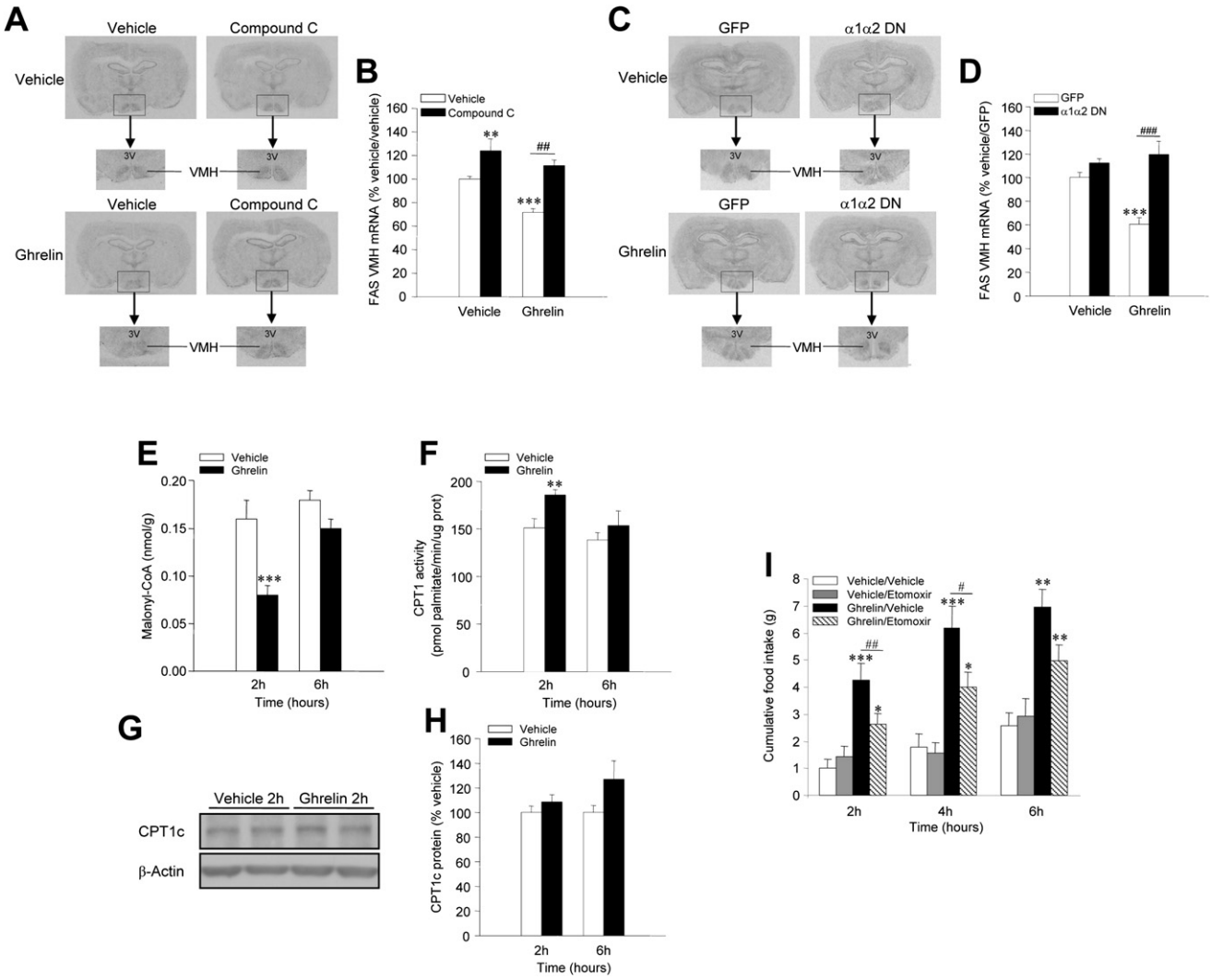

Figure 6. The Orexigenic Effect of Ghrelin Is Mediated by Hypothalamic Malonyl-CoA and CPT1

(A and C) Expression of FAS in the VMH (upper: 5×; lower: 20×) and (B and D) FAS mRNA levels (±SEM) in the VMH of the described groups are shown. Also shown are (E) malonyl-CoA levels (±SEM), (F) CPT1 activity (±SEM), (G) western blot, and (H) CPT1c protein levels (±SEM) in the hypothalamus of vehicle and ghrelin ICV-treated rats. (I) shows cumulative food intake (±SEM) after ICV administration of vehicle or etomoxir prior to ICV administration of vehicle or ghrelin. 3V: third ventricle; *$p < 0.05$ versus vehicle/vehicle; **$p < 0.01$ versus vehicle or vehicle/vehicle; ***$p < 0.001$ versus vehicle or vehicle/vehicle or vehicle/GFP; $^{\#}p < 0.05$ ghrelin/vehicle versus ghrelin/etomoxir; $^{\#\#}p < 0.01$ ghrelin/vehicle versus ghrelin/compound C or ghrelin/etomoxir; $^{\#\#\#}p < 0.001$ ghrelin/GFP versus ghrelin/α1α2 DN.

AMPK Signaling Mediates Ghrelin-Induced Decrease of FAS in the VMH

Our initial data demonstrated that fasting effects on FAS expression are mediated by AMPK-dependent (Figures 1G and 1H) and ghrelin-dependent mechanisms (Figure 5). To investigate the link between those pathways, we analyzed the effect of AMPK inhibition on hypothalamic FAS mRNA expression in ghrelin-treated rats. AMPK inhibition by central administration of compound C to ghrelin-treated rats prevented the ghrelin-induced decrease of FAS mRNA expression in the VMH (Figures 6A and 6B). To further strengthen these data, we used α1α2 DN adenovirus (Woods et al., 2000). Our data demonstrate that stereotaxic delivery of α1α2 DN in the VMH prevented the ghrelin-induced decrease in FAS mRNA levels in the VMH (Figures 6C and 6D). The data demonstrate that the ghrelin effect on FAS mRNA expression is mediated by an AMPK-dependent mechanism.

The Orexigenic Effect of Ghrelin Is Mediated by Decreasing Hypothalamic Content of Malonyl-CoA and Stimulation of Hypothalamic CPT1 Activity

Having shown that ghrelin induces activation of AMPK, phosphorylation of ACC, and decreased FAS levels, we investigated the net effect of these changes in malonyl-CoA. Our results show that hypothalamic malonyl-CoA levels decrease acutely

groups are shown. 3V: third ventricle; *$p < 0.05$ versus vehicle; **$p < 0.01$ versus vehicle; ***$p < 0.001$ versus fed or vehicle; $^{\#}p < 0.05$ fast 24 hr/vehicle versus fast 24 hr/ghrelin antagonist (BIM-28163); $^{\#\#\#}p < 0.001$ versus WT/fast 24 hr.

2 hr after ghrelin treatment, and its levels increase up to normal values by 6 hr after ghrelin treatment (Figure 6E).

Ghrelin regulated hypothalamic CPT1 activity in a time-dependent fashion, and the activity increased acutely at 1 hr (data not shown) and 2 hr and was restored to normal levels by 6 hr after treatment (Figure 6F). Interestingly, the timing of these changes in CPT1 response matched the changes in malonyl-CoA levels induced by ghrelin treatment. Moreover, no changes were detected in the hypothalamic protein levels of CPT1 (Figures 6G and 6H). This suggests that the stimulatory action of ghrelin on CPT1 activity may be mediated by simultaneous changes in malonyl-CoA levels. To determine whether the effect of ghrelin on food intake involved the activation of CPT1, we investigated the effect of etomoxir, an inhibitor of CPT1 (Thupari et al., 2002), on ghrelin orexigenic effect. ICV administration of etomoxir decreased the orexigenic effect of ghrelin at 2 hr (55% decreased compared to ghrelin-treated; $p < 0.01$) and 4 hr (34% decreased compared to ghrelin-treated; $p < 0.05$) after its ICV administration (Figure 6I). The selected dose of etomoxir inhibited CPT1 activity (vehicle-treated: 157.3 ± 18.4 pmol palmitate/min/μg protein versus etomoxir-treated: 109.0 ± 13.1 pmol palmitate/min/μg protein; $p < 0.05$). However, when administered alone, this dose did not have any effect on feeding, excluding the possibility of a direct anorectic effect. Overall, the data demonstrate that ghrelin orexigenic action is partially mediated by stimulation of CPT1 activity.

DISCUSSION

The main conclusion from our study is that the orexigenic effect of ghrelin involves the activation of hypothalamic AMPK and inactivation of enzymatic steps of the de novo fatty acid biosynthetic pathway in the VMH, resulting in decreased malonyl-CoA leading to activation of CPT1 (Figure 7). This observation indicates that VMH fatty acid metabolism is a bona fide component of the energy homeostatic system that integrates peripheral signals, such as ghrelin, with the central mechanisms regulating food intake.

Recent evidence indicates that hypothalamic lipid metabolism plays a role in energy homeostasis through modulation of malonyl-CoA and LCFA-CoA levels (Hu et al., 2003; Obici et al., 2003; He et al., 2006; Pocai et al., 2006; López et al., 2006). Additionally, pharmacological and genetic models have shown that impairment of the fatty acid synthesis pathway in CNS has a severe impact on food intake and body weight homeostasis (Loftus et al., 2000; Obici et al., 2003; Minokoshi et al., 2004; López et al., 2006; Chakravarthy et al., 2007); however the physiological relevance of the hypothalamic fatty acid metabolism in the feeding neurocircuitry and, more importantly, its relationship with peripheral signals regulating food intake remains unknown.

It is widely accepted that the effect of ghrelin on feeding is exerted in the hypothalamus via the ARC where GHS-R mRNA is coexpressed with NPY and AgRP (Seoane et al., 2003; Smith, 2005). Recent data also indicate that ghrelin modulates hypothalamic AMPK (Andersson et al., 2004; Kohno et al., 2008). However, despite this evidence, the molecular mechanisms and anatomical details of this interaction have not been fully identified. More importantly, there is no mechanistic data indicating that AMPK is required for ghrelin's orexigenic effects. In this study,

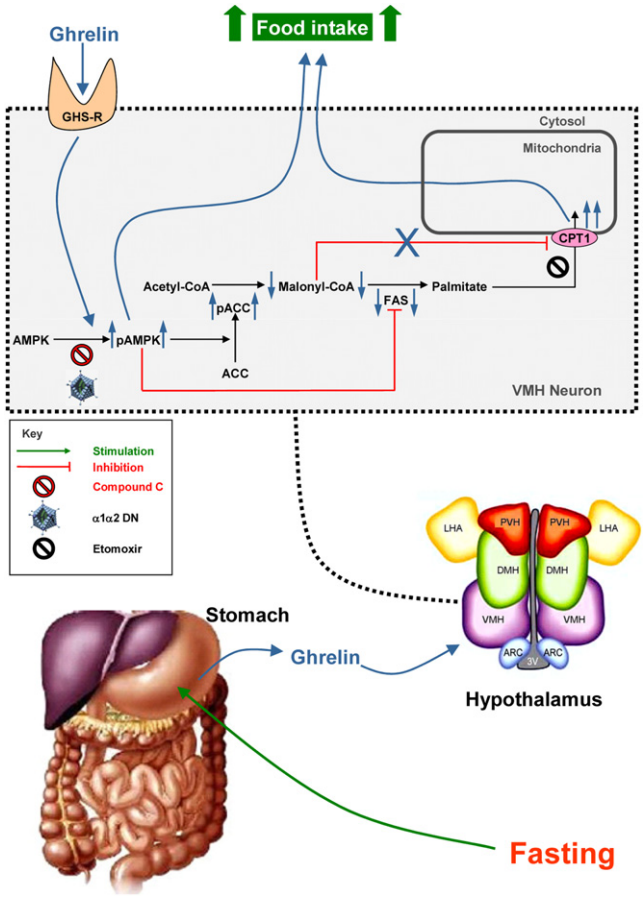

Figure 7. Proposed Model of Ghrelin Actions on Hypothalamic Fatty Acid Metabolism

Fasting increases ghrelin secretion from stomach. Ghrelin orexigenic effects involve the activation of hypothalamic AMPK and inactivation of enzymatic steps of the de novo fatty acid biosynthetic pathway, resulting in decreased malonyl-CoA, leading to activation of CPT1. In addition, fasting downregulates FAS in the VMH through an AMPK and ghrelin-dependent mechanisms. Ghrelin actions are represented by blue arrows and a blue X, which shows attenuation of CPT1 inhibition. 3V: third ventricle; α1α2 DN: AMPKα1 and α2 dominant-negative adenovirus; ARC: arcuate nucleus of the hypothalamus; DMH: dorsomedial nucleus of the hypothalamus; LHA: lateral hypothalamic area; PVH: paraventricular nucleus of the hypothalamus; VMH: ventromedial nucleus of the hypothalamus.

we demonstrate that central inactivation of AMPK in the VMH blocks the orexigenic effect of ghrelin and that this effect is associated with decreased hypothalamic malonyl-CoA content and activation of CPT1.

Levels of malonyl-CoA play a key role in fatty acid turnover by acting both as a substrate for FAS and as an inhibitor of CPT1, the enzyme importing fatty acyl-CoAs into mitochondria for oxidation (Obici et al., 2003; Wolfgang et al., 2006). Recent reports have demonstrated that pharmacological inhibition or gene targeting of CPT1 reduces food intake (Obici et al., 2003; Wolfgang et al., 2006). Our results show that malonyl-CoA is acutely decreased by 2 hr after ghrelin administration, as a result of ghrelin action phosphorylating AMPK and inhibiting ACC. This resulted in a decrease in malonyl-CoA levels associated with increased CPT1 activity, which resulted in increased feeding. The

physiological significance of ghrelin's rapid and transient effect on depleting malonyl-CoA levels and activating CPT1 is intriguing. However, given the potential role of peripheral (stomach-derived) ghrelin in promoting meal initiation (Cummings et al., 2001; Drazen et al., 2006), it is tempting to speculate a potential role for the fasting-induced ghrelin-AMPK-malonyl-CPT1 hypothalamic system as an acute signal, triggering the beginning of feeding after a period of fasting coincident with increased ghrelin levels. Further work using a genetic ablation strategy, such as the CPT1c KO mice (Wolfgang et al., 2006), instead of CPT1's pharmacological inhibition with etomoxir, might help to clarify this hypothesis. One interesting point is the elucidation of the precise roles of both hypothalamic ACC isoforms (ACCα and ACCβ). Despite ACCβ being the isoform mainly involved in the regulation of fatty acid oxidation, at least in nonlipogenic tissues (Wolfgang and Lane, 2006), our data suggest that in the hypothalamus, ACCα may modulate both fatty acid synthesis and oxidation. Another intriguing question is the output system for this mechanism. Current data have demonstrated that the sympathetic nervous system is implicated in the transmission of the "malonyl-CoA signal" from the brain to skeletal muscle (Cha et al., 2005) and also in the modulation of central ghrelin actions on white adipose tissue (WAT) lipid metabolism (Theander-Carrillo et al., 2006). On the basis of these data, a similar mechanism may be involved in the output response of the ghrelin-AMPK-malonyl-CPT1 axis from the hypothalamus to peripheral organs, such as the pancreas, liver, and WAT.

In peripheral tissues, such as liver, muscle, and WAT, the physiological response to starvation involves a coordinated downregulation of the lipogenic pathway with simultaneous activation of the oxidative pathway (Brown and Goldstein, 1997). Lipids are an important component of the CNS composition; the brain relies heavily on lipids to maintain its metabolic and cell membrane homeostasis (Goldberg, 2003). Thus, it would certainly be unexpected to find that the CNS lipid metabolism may be subjected to major changes linked to daily nutritional changes. In fact, it is counterintuitive that physiological periods of food deprivation may compromise lipid metabolism in the entire CNS. However, it cannot be excluded that the mechanisms involved in lipid biosynthesis may, in selected nuclei, be used as a sensing mechanism to maintain energy balance. Our data provide evidence that the effect of starvation on hypothalamic fatty acid metabolism is different than in peripheral organs. For instance, despite the activity of AMPK and ACC being increased in whole hypothalamus during fasting (Minokoshi et al., 2004), levels of FAS mRNA and protein levels are maintained not only in most hypothalamic nuclei (e.g., ARC or PVH) but also in the rest of the CNS areas we studied (cortex, hippocampus, thalamus, etc.). However, we have identified the VMH as the hypothalamic nuclei where FAS is specifically downregulated in response to fasting. Interestingly, our results also indicate that the energy homeostasis-related signal sensed to regulate fatty acid metabolism in the hypothalamus and feeding behavior may not necessarily be a nutrient, but the stomach-secreted hormone ghrelin, acting through hypothalamic AMPK. There is evidence that AMPK also controls the activity of ACC and MCD and the expression of FAS mRNA in peripheral tissues, such as liver and adipose tissue (Saha et al., 2000; Zhou et al., 2001). However, our data, by using both pharmacological and adenovirus-based approaches, show that hypothalamic FAS expression is a downstream target of AMPK in the hypothalamus.

The physiological significance of ghrelin effects on FAS-expressing neurons in the VMH is intriguing. The decrease in malonyl-CoA levels in response to ghrelin is transient so that by 6 hr after ghrelin treatment, malonyl-CoA is restored to its normal levels. Even though the mechanism by which malonyl-CoA levels are restored may be related to the termination of ghrelin stimulation of AMPK, our data also suggest that a direct effect of ghrelin decreasing FAS expression and activity in the VMH may also contribute to this process. Thus, we propose that ghrelin-induced and fasting-induced decreases in FAS levels in the VMH may be a physiological adaptive mechanism that helps to prevent malonyl-CoA from decreasing to deleteriously low levels in the hypothalamus. The observation that the decrease of FAS in response to fasting and ghrelin administration is limited to the VMH is particularly interesting. The VMH is well placed to integrate peripherally supplied signals that regulate food intake with the input from other neighboring nuclei and the brainstem, connected through specific neuronal projections (Tong et al., 2007). Thus, our data provide evidence indicating that, besides its role of maintaining lipid biosynthesis, FAS in the VMH may also have an additional role as a sensor of the nutritional state.

In summary, here we show that the orexigenic action of ghrelin depends on the specific regulation of hypothalamic fatty acid metabolism mediated by AMPK, malonyl-CoA, and CPT1 activity (Figure 7). Our data also identify the fatty acid biosynthetic pathway in the VMH as a potentially important physiological mediator of feeding behavior of relevance for the understanding and treatment of obesity.

EXPERIMENTAL PROCEDURES

Animals

We used adult male Sprague-Dawley rats and lean and obese Zucker rats, adult male GHS-R KO mice, ob/ob mice, and their wild-type (WT) littermates. The experiments were performed in agreement with the International Law on Animal Experimentation. We used 8–16 animals per group, apart from immunohistochemistry where we used 4–6.

Treatments

Fed and fasted rats received two IP administrations of leptin (200 μg). Animals were sacrificed 12 hr after the second injection (9:00 am, day 1), exactly 24 hr after fasting started. Chronic (ICV) cannulae were stereotaxically implanted as previously described (López et al., 2006). Rats received an ICV administration of ghrelin (0.25, 2, or 5 μg; Bachem), ghrelin antagonist BIM-28163 (10 nmol; Ipsen) (Halem et al., 2005), AgRP (5 μg) (Tang-Christensen et al., 2004), NPY (10 μg) (Tritos et al., 1998), etomoxir (10 μg), compound C (10 μg), or vehicle (saline or DMSO). For ghrelin IP treatments rats received a single administration of ghrelin (10 nmol = 33.7 μg) (Wren et al., 2000; Andersson et al., 2004) and were killed after 6 hr. All drugs (apart from ghrelin and BIM-28163) were from Sigma.

Stereotaxic Microinjection of Adenoviral Expression Vectors

Rats were placed in a stereotaxic frame (David Kopf Instruments) under ketamine/xylazine anesthesia. The VMH were targeted bilaterally using a 25-gauge needle (Hamilton) connected to a Hamilton 1 μl syringe. The injection was directed to stereotaxic coordinates 2.3/3.3 mm posterior to the bregma (two injections were performed in each VMH), ± 0.6 mm lateral to midline and 10.2 mm below the surface of the skull (Figure 4A). Adenovirus vectors were one of the following: (1) Cre-GFP (1×10^{10} pfu/ml) or (2) $\alpha1\alpha2$ DN (mix 1:1); they were delivered at a rate of 200 nl/min for 5 min (1 μl/injection site), and the entire injector system was left in place for an additional 5 min after

the injections were completed. After the procedure was complete, chronic (ICV) cannulae were stereotaxically implanted as previously described (López et al., 2006). Six days after adenovirus injection rats were ICV-treated with ghrelin for 6 hr. After that time their brains were dissected and processed by GPF-immunohistochemistry and FAS in situ hybridization or western blot. We used 10 animals per group.

Blood Biochemistry

Plasma, leptin, and ghrelin levels were measured using rat ELISA kits (Crystal Chem Inc).

In Situ Hybridization

Coronal brain sections (16 μm) were probed with specific antisense oligos (Table S2) as previously described (López et al., 2006) (see Supplemental Data).

Immunohistochemistry

Diaminobenzidine (DAB) immunohistochemistry, immunofluorescence, and double labeling were performed as described (López et al., 2006) using a rabbit anti-GFP, anti-FAS (Abcam), and/or anti-GHS-R antibody and/or an anti-BDNF antibody (Santa Cruz) (see Supplemental Data).

Western Blotting

Hypothalamic total protein lysates were subjected to SDS-PAGE, electrotransferred on a PVDF membrane, and probed with the following antibodies: ACC, pACC-Ser79, AMPKα1, AMPKα2, and pIRF-3-Ser396 (Upstate); CaMKKα, CaMKKβ, and pGP (Santa Cruz); CPT1c (Phoenix); FAS (BD), pAMPKα-Thr172, and pSRC-Tyr527 (Cell Signaling); β-Actin and GFP (Abcam) as previously described (López et al., 2006).

Enzymatic Assays

FAS activity was performed as previously described (Lelliott et al., 2005). CPT1 activity was measured using the method of Bieber et al. (1972).

Malonyl-CoA Assay

Malonyl-CoA was measured as previously described (Lelliott et al., 2005; López et al., 2006).

Statistical Analysis

Data are expressed as mean ± SEM. Statistic significance was determined by Student's t test or ANOVA and post-hoc Bonferroni test. $p < 0.05$ was considered significant.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, two tables, and three figures and can be found with this article online at http://www.cellmetabolism.org/cgi/content/full/7/5/389/DC1/.

ACKNOWLEDGMENTS

We thank Dr. Rubén Nogueiras (University of Santiago de Compostela and University of Cincinnati) for his suggestions and criticisms of this paper and Victoria Bonnici for editing the manuscript. This work has been supported by grants from Medical Research Council (A.V.-P. and D.C.), Welcome Trust (A.V.-P.), Xunta de Galicia (C.D.: PGIDIT; M.L.: GRC2006/66; and R.G.: PGIDIT07PXIB208112PR), Fondo Investigationes Sanitarias (M.L.: PI061700), Ministerio de Educacion y Ciencia (C.D.: BFU2005), European Union (C.D. and M.T.: LSHM-CT-2003-503041: Diabesity http://www.eurodiabesity.org; M.L.: Marie Curie Program QLK6-CT-2002-51671; and A.V.-P.: LSHM-CT-2005–018734: Hepadip, http://www.hepadip.org), Mútua Madrileña (C.D. and M.L.), United States Public Health Service (A.K.S.: DK-19514 and DK-67509).

Received: September 23, 2007
Revised: January 30, 2008
Accepted: March 11, 2008
Published: May 6, 2008

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