

# AMP-activated protein kinase plays a role in the control of food intake

Ulrika Andersson<sup>†‡</sup>, Karin Phillipsson<sup>\*‡</sup>, Caroline R. Abbott<sup>\*</sup>, Angela Woods <sup>†</sup>, Kirsty Smith<sup>\*</sup>, Stephen R. Bloom<sup>\*</sup>, David Carling<sup>†‡</sup> and Caroline J. Small<sup>\*‡</sup>.

<sup>†</sup>MRC Clinical Sciences Centre, Cellular Stress Group and <sup>\*</sup>Endocrine Unit, Imperial College London, Hammersmith Campus, Du Cane Rd, London W12 ONN, UK

<sup>‡</sup> Both first and last authors contributed equally to this collaborative study.

## Address for correspondence:

David Carling, MRC Clinical Sciences Centre, Cellular Stress Group, Imperial College London, Hammersmith Campus, Du Cane Rd, London W12 ONN, UK. Tel: 44 (0)208 383 4313; Fax: 44(0)208 383 8306; email: [dcarling@csc.mrc.ac.uk](mailto:dcarling@csc.mrc.ac.uk)

Caroline J. Small, Endocrine Unit, Imperial College London, Hammersmith Campus, Du Cane Rd, London W12 ONN, UK. email: [c.small@imperial.ac.uk](mailto:c.small@imperial.ac.uk)

## **Abstract**

**AMP-activated protein kinase (AMPK) is the downstream component of a protein kinase cascade that acts as an intracellular energy sensor maintaining the energy balance within the cell. The finding that leptin and adiponectin activate AMPK to alter metabolic pathways in muscle and liver provides direct evidence for this role in peripheral tissues. The hypothalamus is a key regulator of food intake and energy balance, coordinating body adiposity and nutritional state in response to peripheral hormones, such as leptin, peptide YY(3-36) (PYY) and ghrelin. To date the hormonal regulation of AMPK in the hypothalamus, or its potential role in the control of food intake, have not been reported. Here we demonstrate that counter-regulatory hormones involved in appetite control regulate AMPK activity, and that pharmacological activation of AMPK in the hypothalamus increases food intake. In vivo administration of leptin, which leads to a reduction in food intake, decreases hypothalamic AMPK activity. By contrast, injection of ghrelin in vivo, which increases food intake, stimulates AMPK activity in the hypothalamus. Consistent with the effect of ghrelin, injection of 5-amino-4-imidazole carboxamide (AICA) riboside, a pharmacological activator of AMPK, into either the third cerebral ventricle or directly into the paraventricular nucleus of the hypothalamus significantly increased food intake. These results suggest that AMPK is regulated in the hypothalamus by hormones which regulate food intake. Furthermore, direct pharmacological activation of AMPK in the hypothalamus is sufficient to increase food intake. These findings demonstrate that AMPK plays a role in the regulation of feeding and identify AMPK as a novel target for anti-obesity drugs.**

## **Introduction**

AMP-activated protein kinase (AMPK) plays a pivotal role in the regulation of energy metabolism and has been dubbed a cellular fuel gauge (1). AMPK is activated following an increase in the AMP:ATP ratio within the cell that occurs following a decrease in ATP levels (2,3). Once activated, AMPK switches on ATP-generating (catabolic) pathways e.g. fatty acid oxidation, and switches off ATP-using pathways (anabolic) pathways e.g. fatty acid synthesis, allowing the cell to restore its energy balance (2,3). In addition to acute effects on metabolism, AMPK has more long-term effect, altering both gene (4) and protein expression (5,6). Recent results have demonstrated activation of AMPK in the absence of changes in adenine nucleotide levels, indicating that there may be multiple pathways upstream of AMPK (7,8). The molecular mechanisms leading to activation of AMPK have not been fully elucidated, but it is clear that activation of AMPK requires phosphorylation of threonine 172 (T172) within the activation loop segment of the catalytic ( $\alpha$ ) subunit (9,10). Very recently, LKB1, a protein kinase that is inactivated in a hereditary form of cancer termed Peutz-Jeghers syndrome, was shown to account for most of the AMPK kinase activity in cell extracts (11,12) raising the possibility that AMPK could link metabolism with cell proliferation.

Until fairly recently, most of the studies examining the role of AMPK have focussed on its response to acute changes in energy levels within individual cells. However, there is emerging evidence that AMPK also plays an important role in the regulation of whole-body energy metabolism, responding to adipocyte-derived hormones such as leptin (13) and adiponectin (14). Leptin activates AMPK in skeletal muscle increasing fatty acid oxidation (13), whilst adiponectin activates AMPK in both liver and skeletal muscle,

increasing glucose utilisation and fatty acid oxidation, and inhibiting glucose production in the liver (14).

The hypothalamus and the dorsal vagal complex appear to be the main regions within the central nervous system (CNS) directly regulating appetite. The arcuate nucleus and the paraventricular nucleus (PVN) of the hypothalamus have been shown to play an integrative role in appetite regulation (15). Neurones within the hypothalamus respond to the different neuro-endocrine and metabolic signals co-ordinating the body's response to changes in energy intake and energy expenditure. The mechanisms involved are complex but depend at least in part on hormones derived from either adipose tissue, e.g. leptin or the gastrointestinal tract e.g. ghrelin. Leptin, a hormone derived from adipocytes, acts on neurones within the arcuate nucleus of the hypothalamus, decreasing the release of orexigenic neuropeptides and increasing the release of anorexigenic neuropeptides, resulting in decreased food intake (15). Ghrelin is synthesised in the stomach and stimulates food intake, acting at least in part via the same neuronal circuits involved in the response to anorexigenic neuropeptides (15). Thus, leptin and ghrelin have counter-regulatory effects on food intake, although the cellular mechanisms by which they act are poorly understood. The aim of the current study was to determine whether AMPK in the hypothalamus plays a role in the regulation of food intake.

## **Experimental Procedures**

### ***Materials***

Ghrelin, Neuropeptide Y (NPY) and Peptide YY(3-36) were all purchased from Bachem UK Ltd (Merseyside, UK). Leptin was purchased from R & D (source). 5-amino-4-imidazole carboxamide riboside was from Sigma.

### ***Animals***

Male Wistar rats (180-220 g) were maintained in individual cages under controlled temperature (21-23°C) and light (12 h light (7am-7pm), 12 h dark) conditions with *ad libitum* access to food (RM1 diet, SDS UK Ltd) and water. Animals were handled daily following recovery from surgery until the completion of the studies. All animal procedures undertaken were approved by the British Home Office Animals Scientific Procedures Act 1986.

### ***Intraperitoneal injection***

Rats were accustomed to the intraperitoneal injection procedure by injection of 0.5 ml saline and the measurement of food intake two days prior to the study. Where appropriate, rats received an intraperitoneal injection of saline, leptin (1.1 mg/kg), ghrelin (30 nmole/animal) or PYY(3-36) (25 nmole/animal) in a total volume of 0.5 ml. Injections were given at the start of the light phase and in the case of leptin and PYY animals were fasted for 12 hours prior to injection.

### ***Intracerebroventricular (ICV) and inter paraventricular nucleus (PVN) cannulation and injection***

Animal surgical procedures and handling were carried out as previously described (16,17).

Animals were anaesthetized by intraperitoneal injection of a mixture of Ketalar (ketamine HCl 60 mg/kg, Parke-Davis, Pontypool, UK) and Rompun (xylazine 12 mg/kg, Bayer UK Ltd, Bury St. Edmunds, UK) and placed in a Kopf stereotaxic frame. Permanent 26 gauge stainless steel guide cannulae (Plastics One Inc. Roanoke, VA) were stereotactically placed 1.8mm posterior to bregma, 0.5mm lateral from the mid-sagittal line and implanted 7mm below the outer surface of the skull into the paraventricular nucleus of the hypothalamus. The third cerebral ventricle was cannulated with a permanent 22 gauge stainless steel guide cannula (Plastics One inc.

Roanoke, VA, USA) stereotactically placed 0.8mm posterior to bregma on the mid-line and implanted 6.5mm below the outer surface of the skull. All animals used in the study were mock injected on two occasions to acclimatise them to the procedure prior to the first study day. Substances were administered via a stainless steel injector placed in, and projecting 1mm below, the tip of the guide cannulae. All compounds were dissolved in 0.9% saline and injected in a volume of 1µl (PVN) and 5µl (ICV). The entire injection process lasted under 2 minutes, and the rats were returned to their cages with the minimum of disruption. Correct placement of the cannula into the third cerebral ventricle was confirmed by injection of Angiotensin II (150ng) as previously described (18). Animals not displaying a prompt and sustained drinking response were excluded from further study. This was approximately 5% of cannulated animals. For intranuclear cannulated rats correct cannula placement was confirmed histologically at the end of the study period, as previously described (16,19). Following injection of black ink, animals were decapitated, the guide cannulae removed and the brains immediately frozen in liquid nitrogen and stored at -70°C. Brains were sliced on a cryostat (Bright Instruments, Huntingdon, Cambridgeshire, UK) into 15 µM coronal sections and stained with cresyl violet. Sections were compared with the corresponding section from the rat brain atlas. The ink remained localised at the injection site at the guide tip without significant diffusion. Data from an animal was excluded if its injection site extended more than 0.2 mm outside the nucleus or if any ink was detected in the cerebral ventricular system. Consistent with previously reported studies (16,19) approximately 12% of intranuclear cannulated animals were excluded for incorrect cannula placement.

### ***Isolation of tissues***

Animals were sacrificed by decapitation, brains immediately dissected and the hypothalamus removed and snap-frozen in liquid nitrogen. Frozen tissues (approximately 50 mg) were homogenised in 0.2 ml ice-cold 50 mM Tris/HCl, pH 7.5, 50 mM NaF, 5 mM Na pyrophosphate, 250 mM sucrose, 1 mM EDTA, 1 mM DTT, 1 mM benzamidine, 0.1% (w/v) phenylmethylsulphonylfluoride using an UltraTurax homogeniser (3 x 30 s bursts). Insoluble material was removed by centrifugation and the resulting supernatant used for immunoprecipitation of AMPK and Western blot analysis.

### ***AMPK assay***

AMPK was immunoprecipitated from 100 µg total protein using an anti-pan β antibody (20) bound to protein A-Sepharose and activity measured by phosphorylation of the SAMS synthetic peptide (21).

### ***Western blot analysis***

Tissue lysates (40 µg) were resolved by SDS-PAGE and transferred to polyvinylidene fluoride membranes. Phosphorylation of AMPK was determined by blotting with a phosphothreonine 172 specific antibody (Cell Signalling Technologies). Total AMPK was estimated by blotting with an anti-pan β antibody (20). ACC phosphorylation was measured with a phospho-ACC specific antibody (Upstate). In each case, anti-rabbit antibody linked to horse radish peroxidase (Bio-Rad) was used as the secondary antibody. Total ACC was determined using streptavidin linked to horse radish peroxidase (Bio-Rad). Blots were developed using enhanced chemiluminescence (Pierce).

## Results and Discussion

Intraperitoneal injection of leptin (1.1 mg/kg body weight) caused a time-dependent decrease in AMPK activity in the hypothalamus (Fig. 1a). Forty minutes following injection there was no decrease in AMPK activity, but 60 minutes after injection AMPK activity was reduced by 25-30% ( $n=10$ ,  $p<0.005$ ). The reduction in AMPK activity persisted for up to 180 minutes ( $p<0.05$ ), but returned to control values after 300 minutes. Consistent with the decrease in AMPK activity, the phosphorylation state of acetyl-CoA carboxylase (ACC), a downstream target of AMPK (13), isolated from the hypothalamus was reduced at both 60 and 180 minutes following leptin administration (Fig. 1b). AMPK is activated by phosphorylation of threonine 172 (T172) within the activation loop of the catalytic ( $\alpha$ ) subunit of AMPK (10). This phosphorylation is catalysed by an upstream kinase, which was recently identified as LKB1, a kinase that is inactivated in a hereditary cancer termed Peutz-Jeghers syndrome (11,12). In vivo administration of leptin led to a slight reduction in LKB1 activity, although this did not reach statistical significance (Fig. 1c), and a reduction in the phosphorylation state of T172 (Fig. 1d).

In contrast to the effects of leptin, intraperitoneal injection of ghrelin (30 nmole/animal) activated AMPK in the hypothalamus (Fig. 2a). The dose of ghrelin we used has previously been shown to stimulate food intake one hour after administration (17). Similar to the effect of leptin, 40 minutes following injection of ghrelin there was no change in AMPK activity, whereas 60 minutes after injection AMPK activity was increased by 45-50% ( $n=10$ ,  $p<0.05$ ). AMPK activity returned to control levels after 180 minutes. In parallel with the activation of AMPK there was an increase in the phosphorylation state of ACC 60 minutes following



injection, but no change after 180 minutes (Fig. 2b). LKB1 activity in the hypothalamus was increased, but this did not reach statistical significance (Fig. 2c).

Peptide YY(3-36) (PYY) is a gastrointestinal tract-derived hormone that is released post-prandially in proportion to the amount of calories ingested and leads to a reduction in food intake in rodents and man (22). However, unlike leptin and ghrelin we did not detect an effect of PYY on AMPK activity or phosphorylation, or on ACC phosphorylation (data not shown) suggesting that PYY does not regulate AMPK in the hypothalamus.

We next examined the effect of altering AMPK activity in the hypothalamus on food intake. 5-amino-4-imidazolecarboxamide (AICA) riboside can be taken up by cells and converted into AICA ribotide, or ZMP, which acts as an AMP mimetic, and activates AMPK (23). Rats were injected with AICA riboside either into the third cerebral ventricle (ICV) or into the paraventricular nucleus (PVN) and food intake measured over the following 8 hours. Both treatments led to a significant increase in food intake (Fig. 3).

Recently there has been considerable speculation that AMPK might play a role in the CNS regulation of energy metabolism (see for example (24)). Our results provide the first evidence that pharmacological activation of AMPK in the hypothalamus stimulates food intake.

Furthermore, AMPK activity in the hypothalamus is regulated by leptin and ghrelin, hormones that have opposing effects on food intake. In contrast to its effect in skeletal muscle, leptin decreased AMPK activity in the hypothalamus, whereas ghrelin increased AMPK activity. The mechanism(s) by which leptin and ghrelin regulate AMPK are unknown, although our results suggest that this may involve changes in the activity of LKB1, the kinase

immediately upstream of AMPK. Concomitant with their effects on AMPK, leptin decreased ACC phosphorylation and ghrelin increased ACC phosphorylation. A predicted consequence of these effects is that the level of malonyl-CoA would be increased by leptin and decreased by ghrelin. Injection of C75, a synthetic inhibitor of fatty acid synthase, into mice leads to reduced food intake and reduced body weight through a mechanism that is thought to involve malonyl-CoA (25). A recent report demonstrated that inhibition of carnitine palmitoyltransferase 1 (CPT 1), which is inhibited physiologically by malonyl-CoA, reduces food intake (26). Our results are consistent with a model that would involve hormonal-induced changes in AMPK activity leading to changes in ACC phosphorylation activity which in turn would alter malonyl-CoA levels to regulate food intake. In support of such a mechanism is our finding that pharmacological activation of AMPK in the hypothalamus increases food intake. Taken together, these results provide the first demonstration that AMPK is directly involved in regulating food intake and implicate it as a potential target for therapeutic agents aimed at reducing body weight.

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## Figure Legends

### Figure 1. Leptin reduces AMPK activity in the hypothalamus.

Rats were fasted for 12 hours and then injected intraperitoneally with either leptin (1.1 mg/kg body weight) or saline and hypothalami removed 40, 60, 180 and 300 minutes following injection. **(a)** AMPK activity was measured in immune complexes isolated by immunoprecipitation of total tissue homogenate (100 µg total protein) using a pan-AMPK $\beta$  antibody. Results shown are the mean values  $\pm$ S.E.M. (n=5 for 40 minute time-point, n=10 for the other time-points) and are plotted as a percentage of the activity present in tissue isolated from saline treated animals (black bars). Significant differences in AMPK activity between leptin treated and saline control are represented by \* ( $p<0.05$ ). **(b)** Acetyl-CoA carboxylase (ACC) phosphorylation was determined by Western blotting using a phospho-specific antibody (P-ACC). In parallel, total ACC expression was determined using horse radish peroxidase-streptavidin conjugate. Representative blots for two independent tissue samples are shown. **(c)** LKB1 activity in hypothalami isolated 60 minutes after injection was measured in anti-LKB1 immunoprecipitates by activation of recombinant AMPK. Results shown are the mean  $\pm$ S.E.M. from 10 animals and are plotted as a percentage of the activity in saline treated animals. **(d)** AMPK phosphorylation (P-AMPK) 60 minutes after infection of leptin was determined by Western blotting using an antibody specific for phosphothreonine

172 within the catalytic  $\alpha$  subunit. Total AMPK was determined by blotting with an anti-AMPK $\beta$  subunit antibody. Representative blots for two independent samples are shown.

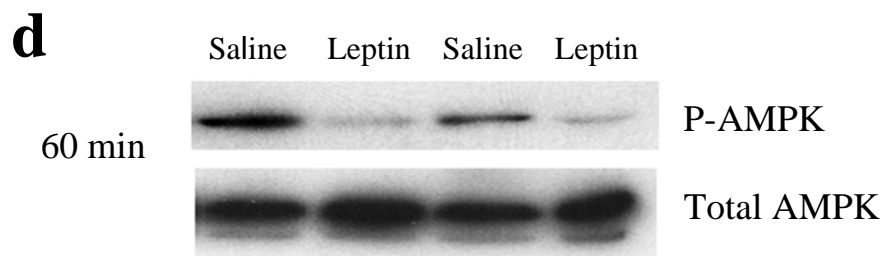
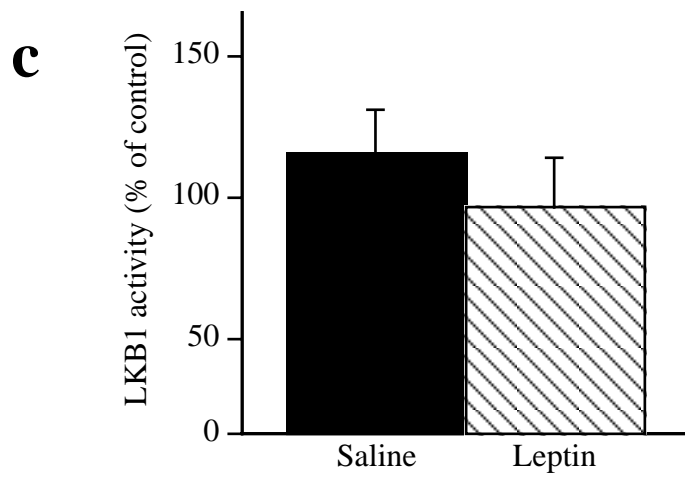
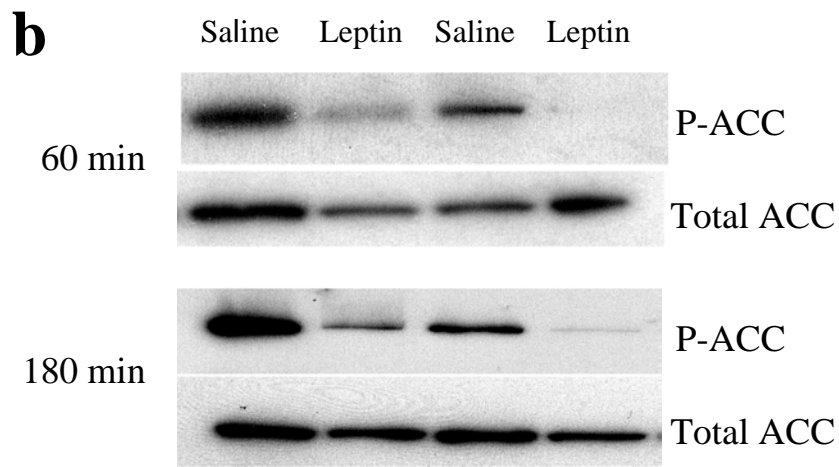
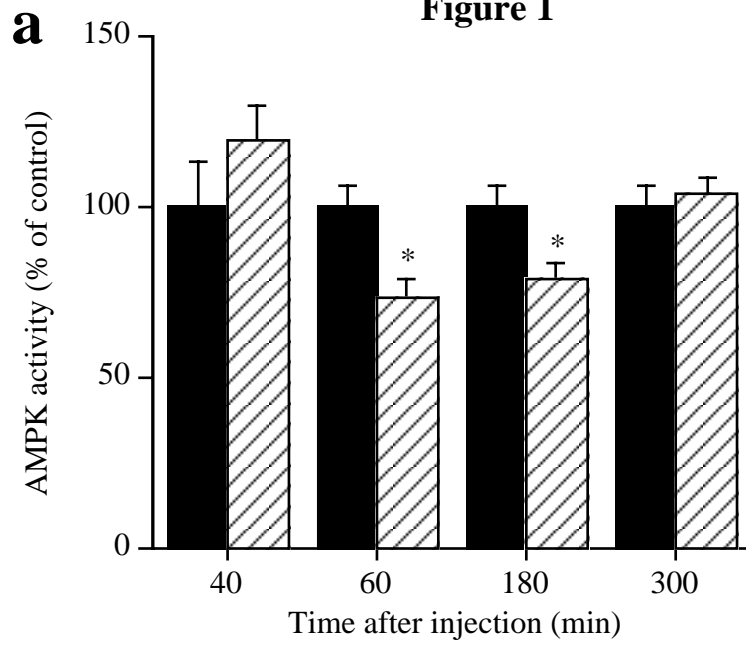
**Figure 2. Ghrelin activates AMPK in the hypothalamus.**

Rats were injected intraperitoneally with either ghrelin (30 nmole/animal) or saline and hypothalami removed after 40, 60, and 180 minutes. **(a)** AMPK activity (mean  $\pm$ S.E.M., n=5 for 40 minute time-point, n=10 for 60 and 180 minute time-points, \*  $p<0.05$ ) **(b)** ACC phosphorylation, **(c)** LKB1 activity (n=10, 60 minutes after injection of ghrelin), and **(d)** AMPK phosphorylation (60 minutes after injection of ghrelin) were determined as described in the legend for Figure 1.

**Figure 3. Activation of AMPK in the hypothalamus increase food intake *in vivo*.**

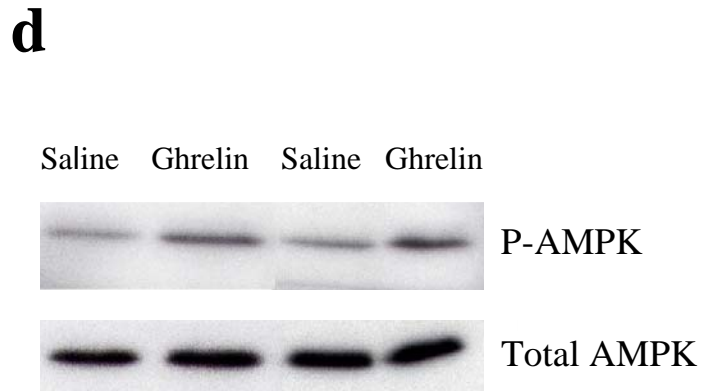
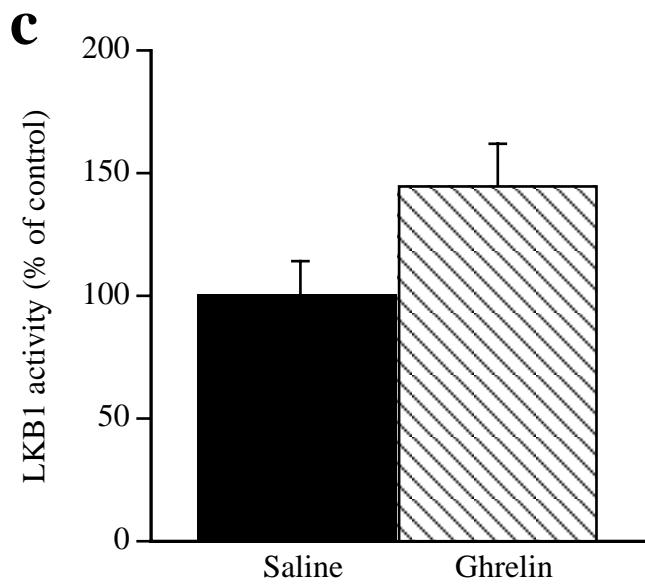
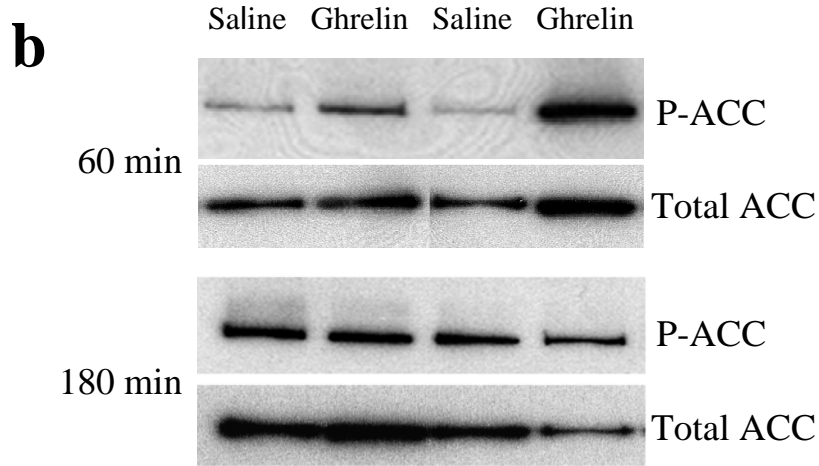
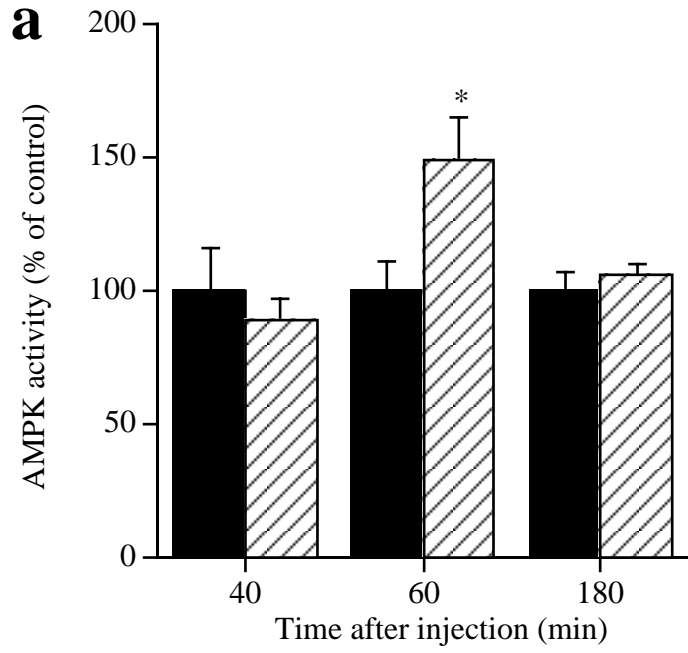
Rats were implanted with permanent indwelling cannulae and following surgical recovery were injected with AICA riboside (closed circles), neuropeptide Y (NPY, open circles) or saline (open squares) and food intake measured over the following 8 hours. **(a)** Intracerebroventricular (ICV) injections were given (1  $\mu$ mole AICA riboside, 5 nmole NPY). Values are the mean  $\pm$ S.E.M. for 7-11 animals. **(b)** Injections were given directly into the paraventricular nucleus (PVN; 10 nmole AICA riboside, 0.5 nmole NPY). Values are the mean  $\pm$ S.E.M. for 11-15 animals. Significant differences in food intake compared to saline control animals are indicated by \* ( $p<0.05$ ).

**Figure 1**



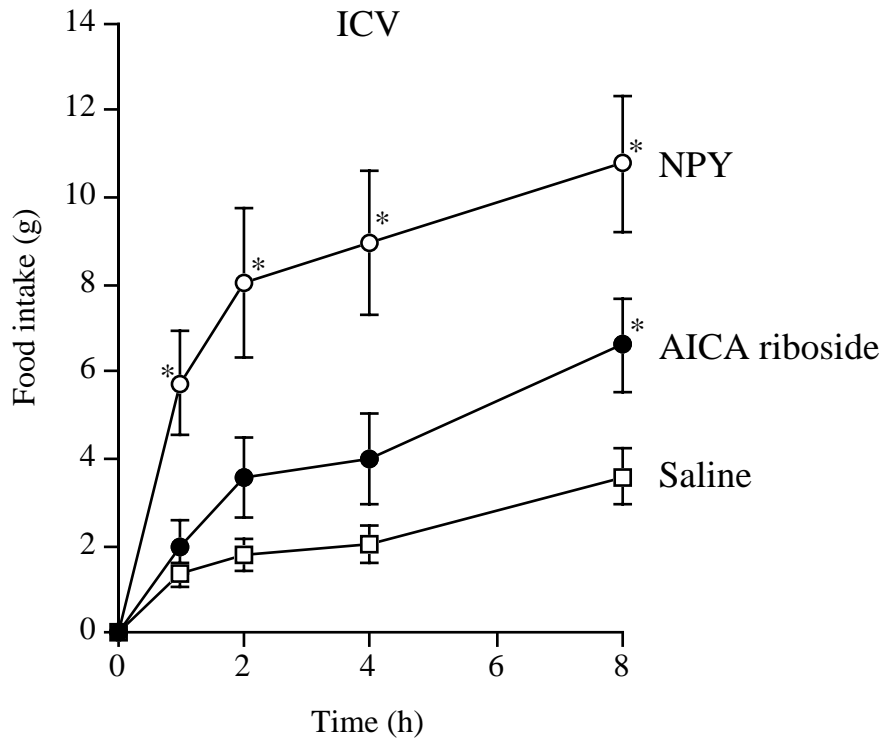


**Figure 2**



**Figure 3**

**a**



**b**

