Diabetologia (2012) 55:215–225
DOI 10.1007/s00125-011-2332-0

ARTICLE

Amylin and leptin activate overlapping signalling pathways in an additive manner in mouse GT1-7 hypothalamic, C_2C_{12} muscle and AML12 liver cell lines

H.-S. Moon · J. P. Chamberland · C. S. Mantzoros

Received: 19 July 2011 / Accepted: 19 September 2011 / Published online: 14 October 2011

Abstract
Aims/hypothesis It has been suggested that amylin amplifies leptin's effects and affects energy homeostasis synergistically with leptin in animals and humans. However, no previous study has reported on amylin and leptin signalling in hypothalamic, muscle and liver cells.
Methods Leptin and amylin signalling studies were performed in vitro in mouse GT1-7 hypothalamic, C_2C_{12} muscle and AML12 liver cell lines.
Results Treatment of mouse GT1-7 and C_2C_{12} cells with leptin or amylin increased signal transducer and activator of transcription 3 (STAT3) phosphorylation in a dose- and time-dependent manner. In mouse AML12 cells, leptin and amylin produced a biphasic response of STAT3 activity. Importantly, all leptin and amylin signalling pathways were saturable at leptin and amylin concentrations of ~100 and ~50 to 100 ng/ml, respectively. Leptin and amylin in combination activated STAT3, AMP-activated protein kinase (AMPK), extracellular signal-regulated kinase (ERK) 1/2 and Akt signalling pathways in an additive manner, effects that were abolished under endoplasmic reticulum (ER) stress. Leptin, but not amylin, increased IRS-1 phosphorylation in GT1-7 hypothalamic, but not in C_2C_{12} muscle and AML12 liver cell lines.
Conclusions/interpretation Our data suggest that leptin and amylin have overlapping and additive, but not synergistic, effects in the activation of intracellular signalling pathways. ER stress may induce leptin and amylin resistance in hypothalamic, muscle and liver cell lines. These novel insights into the mode of action of leptin and amylin suggest that these hormones may play an additive role in regulating energy homeostasis in humans.

Keywords Amylin · Endoplasmic reticulum stress · Hypothalamus · Intracellular signalling pathway · Leptin · Liver · Muscle

Abbreviations

AMPK	AMP-activated protein kinase
eIF2	Eukaryotic initiation factor 2
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
HRP	Horseradish peroxidase
PI3-kinase	Phosphatidylinositol 3-kinase
siRNA	Small interfering RNA
ssiRNA	Scrambled-siRNA
STAT3	Signal transducer and activator of transcription 3

Introduction

Leptin not only activates central nervous system networks that suppress appetite [1–5], but also acts in the periphery to

Electronic supplementary material The online version of this article (doi:10.1007/s00125-011-2332-0) contains peer-reviewed but unedited supplementary material, which is available to authorised users.

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alter immune function and metabolism [6]. Leptin administration to leptin-deficient (*ob/ob*) mice has been shown to improve insulin resistance even before body weight is reduced, a finding consistent with a direct effect on the activation of signalling pathways that overlap with those of insulin [7, 8]. Amylin is a 37-amino-acid peptide hormone, which is co-secreted with insulin from pancreatic beta cells [9, 10]. It has also been proposed that amylin may act as a leptin sensitiser and that treatment with a combination of amylin and leptin may have synergistic effects in the treatment of obesity in animals [6] and humans [10], affecting weight and fat loss [11, 12].

We have previously demonstrated for the first time that amylin has a direct effect on human adipose tissue and human peripheral blood mononuclear cells in vitro and ex vivo, and that, with leptin, it has additive, but not synergistic, effects on the activation of intracellular signalling pathways in human peripheral tissues [5]. No previous study has investigated amylin- and/or leptin-activated signalling pathways in hypothalamic, muscle and liver cells. To gain better insights into the effects of leptin and amylin in cells that are apparently impossible or difficult to study directly in humans, such as hypothalamic, muscle or liver cells, we performed leptin and amylin signalling studies in vitro, with a focus on their potential interactions. We first studied leptin and amylin signalling in vitro with regard to signal transducer and activator of transcription 3 (STAT3), AMP-activated protein kinase (AMPK), extracellular signal-regulated kinase (ERK)1/2 and Akt activation in mouse GT1-7 hypothalamic, C_2C_{12} muscle and AML12 liver cell lines. Next, we directly measured the phosphorylation of IRS-1 and IRS-2 in response to leptin and amylin, alone or in combination, in the same cell lines by analysing phosphotyrosine levels of IRS-1 or IRS-2 immunoprecipitated with IRS-1 or IRS-2. We then proceeded to examine whether amylin could function as a leptin sensitiser to overcome endoplasmic reticulum (ER) stress, which has previously been described to affect leptin signalling leading to leptin resistance [13, 14].

Methods

Materials Primary and secondary antibodies such as mouse monoclonal STAT3, goat polyclonal phosphorylated STAT3, mouse monoclonal AMPK, goat polyclonal phosphorylated AMPK, mouse monoclonal Akt, rabbit polyclonal phosphorylated Akt, rabbit polyclonal ERK1/2, goat polyclonal phosphorylated ERK1/2, rabbit polyclonal IRS-1, rabbit polyclonal phosphorylated IRS-1, rabbit polyclonal IRS-2, rabbit polyclonal phosphorylated IRS-2, mouse monoclonal eukaryotic initiation factor 2 (eIF2), rabbit polyclonal phosphorylated eIF2, mouse monoclonal beta-actin, anti-rabbit IgG horseradish peroxidase (HRP), anti-mouse IgG HRP and anti-rabbit IgG HRP were purchased from Santa Cruz Biotech (San Francisco, CA, USA). Leptin mouse recombinant was purchased from ProSpec (East Brunswick, NJ, USA). Amylin mouse recombinant was purchased from Phoenix Pharmaceuticals (Burlingame, CA, USA).

Cell culture The mouse GT1-7 hypothalamic cell line was generously provided by Y.-B. Kim, Beth Israel Deaconess Medical Center, Harvard Medical School. The mouse C_2C_{12} muscle and AML12 liver cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in six-well plates and maintained in DMEM containing glucose at 4.5 mg/ml (Sigma) and supplemented with 10% (vol./vol.) heat-inactivated fetal bovine serum (Atlantic Biologicals, Miami, FL, USA). All cells were incubated at 37°C in an atmosphere of 5% CO_2 in air, and subcultured beyond 80% confluence.

Cell proliferation assay A cell proliferation assay was performed using the MTT assay as described by Invitrogen (Carlsbad, CA, USA).

Introduction of small interfering RNA The small interfering RNA (siRNA) and scrambled-siRNA (ssiRNA) of *Stat3* and *Ampk* (also known as *Prkaa1*) were purchased from Santa Cruz. The cells were seeded on to six-well tissue culture plates and grown for 24 h in the absence of antibiotics to 60% confluence before transfection. Immediately prior to transfection, the culture medium was removed and the cells were washed once with PBS, then transfected with siRNA or ssiRNA using a transfection reagent (Fugene 6) in Opti-MEM medium according to the manufacturer's instructions (Roche, Indianapolis, IN, USA). After transfection, cultures were incubated for 5 h at 37°C and then placed in fresh culture medium. Cells were studied after an additional 24 h.

Endoplasmic reticulum stress induction The induction of ER stress was established according to the method described by Hagiwara et al. [15]. Briefly, to induce ER stress, the cells were pre-treated for 5 h with tunicamycin (3 μg/ml) and/or dithiothreitol (1 mmol/l) and subsequently subjected to leptin and/or amylin treatment.

Immunoprecipitation The immunoprecipitation method was established as described previously [16]. Briefly, the cells were cultured as described in detail above (Cell culture). The lysates were adjusted to 1 ml in lysis buffer before addition of the specific antisera used to immunoprecipitate the β-subunit of IRS-1 and IRS-2. The immuno-

precipitations were carried out for 1.5 h and at 4°C in reactions containing 2.5 mg cell lysate and 2.5 μg antiserum. Protein A or protein G sepharose beads (25 μl) were then added for an additional 1.5 h of incubation, followed by two washes of the immunoprecipitates in lysis buffer. The proteins were transferred to membrane and probed with specific antibodies, followed by incubation with an anti-rabbit or anti-mouse IgG conjugated to HRP.

Western blotting The proteins were loaded in each lane. After SDS-PAGE, the proteins were blotted on to nitrocellulose membranes (Schleicher and Schuell, Keene, NH, USA). The membranes were blocked for 1 h in TBS containing 5% (wt/vol.) non-fat dry milk and 0.1% (vol./vol.) Tween-20. Incubation with primary antibodies was performed overnight in TBS containing 5% (wt/vol.) non-fat dry milk, followed by incubation with HRP secondary antibodies for 2 h. After incubation with antibodies, membranes were washed with TBS containing 0.1% (vol./vol.) Tween-20. Enhanced chemiluminescence was used for detection. Measurement of signal intensity on nitrocellulose membranes after western blotting with various antibodies was performed using Image J processing and analysis software (http://rsbweb.nih.gov/ij/, accessed 25 August 2011).

Statistical analysis All signalling data were analysed using one-way ANOVA followed by a post hoc test for multiple comparisons. All analyses were performed using SPSS version 11.5 (SPSS, Chicago, IL, USA).

Results

Dose– and time–response curves in mouse GT1-7 hypothalamic, C_2C_{12} muscle and AML12 liver cell lines Treatment of GT1-7, C_2C_{12} and AML12 cells with 10 ng/ml leptin had no effect on STAT3 activation compared with control (Fig. 1a, c, e). By contrast, administration of 10 ng/ml amylin in GT1-7 cells, but not in C_2C_{12} and AML12 cells, increased STAT3 phosphorylation by ~40% compared with control (Fig. 1a, c, e). Importantly, all leptin and amylin signalling pathways were saturable at leptin and amylin concentrations of ~100 and ~50 to 100 ng/ml, respectively. Leptin and amylin administration also significantly induced phosphorylation of STAT3 in a time-dependent manner, with phosphorylation increased by ~3.9- and ~3.7-fold compared with control at 45 to 90 min in GT1-7 and C_2C_{12} cell lines, respectively (Fig. 1b, d). In AML12 cells, leptin and amylin produced a biphasic response in STAT3 activity with a ~2.5-fold rise at about 10 min, a return to baseline by 30 min and a second ~2.7-fold elevation by 90 min (Fig. 1f). It has been shown that 0.1 ng/ml leptin activates intracellular signalling pathways in mouse muscle cell lines [17]. Since the lowest concentration of leptin and amylin used in the present paper was 10 ng/ml, it is clearly possible that a biphasic effect of leptin and amylin may occur between 0.1 and 10 ng/ml in mouse cell lines. Indeed, we observed that in C_2C_{12} cells leptin and amylin produced a biphasic response in STAT3 activity, with a ~1.7-fold rise at 0.1 ng/ml, a ~2.3-fold rise at 0.5 ng/ml, a ~1.6-fold rise at 1 ng/ml and a return to baseline at 10 ng/ml (electronic supplementary material [ESM] Fig. 1a, b). By contrast, we did not observe any biphasic response in STAT3 activation with low physiological concentrations of leptin or amylin in GT1-7 and AML12 cells (ESM Fig. 1a, b).

Leptin and amylin alone or in combination increase cell proliferation in mouse GT1-7 hypothalamic, C_2C_{12} muscle and AML12 liver cell lines Treatment of mouse cell lines with leptin and amylin alone increased cell proliferation compared with control. These effects were further increased by co-administration of leptin and amylin compared with leptin and/or amylin administration alone (ESM Fig. 2).

Activation of STAT3, AMPK, ERK1/2 and Akt signalling by administration of leptin and amylin alone or in combination in mouse GT1-7 hypothalamic, C_2C_{12} muscle and AML12 liver cell lines Based on the above (Fig. 1), we chose a representative administration time (45 min) and leptin (50 ng/ml) and amylin (20 ng/ml) concentrations, and performed leptin and/or amylin signalling studies. Either leptin or amylin increased phosphorylation of STAT3 compared with control, namely by ~3.6-fold in GT1-7 (Fig. 2a), by ~3.2-fold in C_2C_{12} (Fig. 2b) and by ~2.8-fold in AML12 (Fig. 2c) cell lines. Similarly, either leptin or amylin stimulated AMPK signalling compared with control, with increases of ~3.8-fold in GT1-7 (Fig. 2a), ~3.1-fold in C_2C_{12} (Fig. 2b) and around fourfold in AML12 (Fig. 2c) cell lines. Moreover, in all mouse cell lines, co-administration of leptin and amylin further activated STAT3 and AMPK signalling compared with leptin or amylin administration alone (Fig. 2a–c). By contrast, increased STAT3 and/or AMPK phosphorylation by leptin + amylin administration was abolished by *Stat3* and/or *Ampk* siRNA administration in all mouse cell lines (Fig. 3a, b). We also observed that administration of *Stat3* and/or *Ampk* siRNA blocked the production of total STAT3 and AMPK in all mouse cell lines (data not shown). We then proceeded to study the activation of ERK1/2 and Akt signalling pathways in GT1-7, C_2C_{12} and AML12 cell lines. We found that leptin or amylin increased phosphorylation of ERK1/2 compared with control in all mouse cell lines (Fig. 4a). Leptin and amylin also stimulated Akt signalling compared with control in all mouse cell lines (Fig. 4b). Moreover, in all mouse cell lines, co-administration of leptin and amylin further activated ERK1/2 and Akt signalling

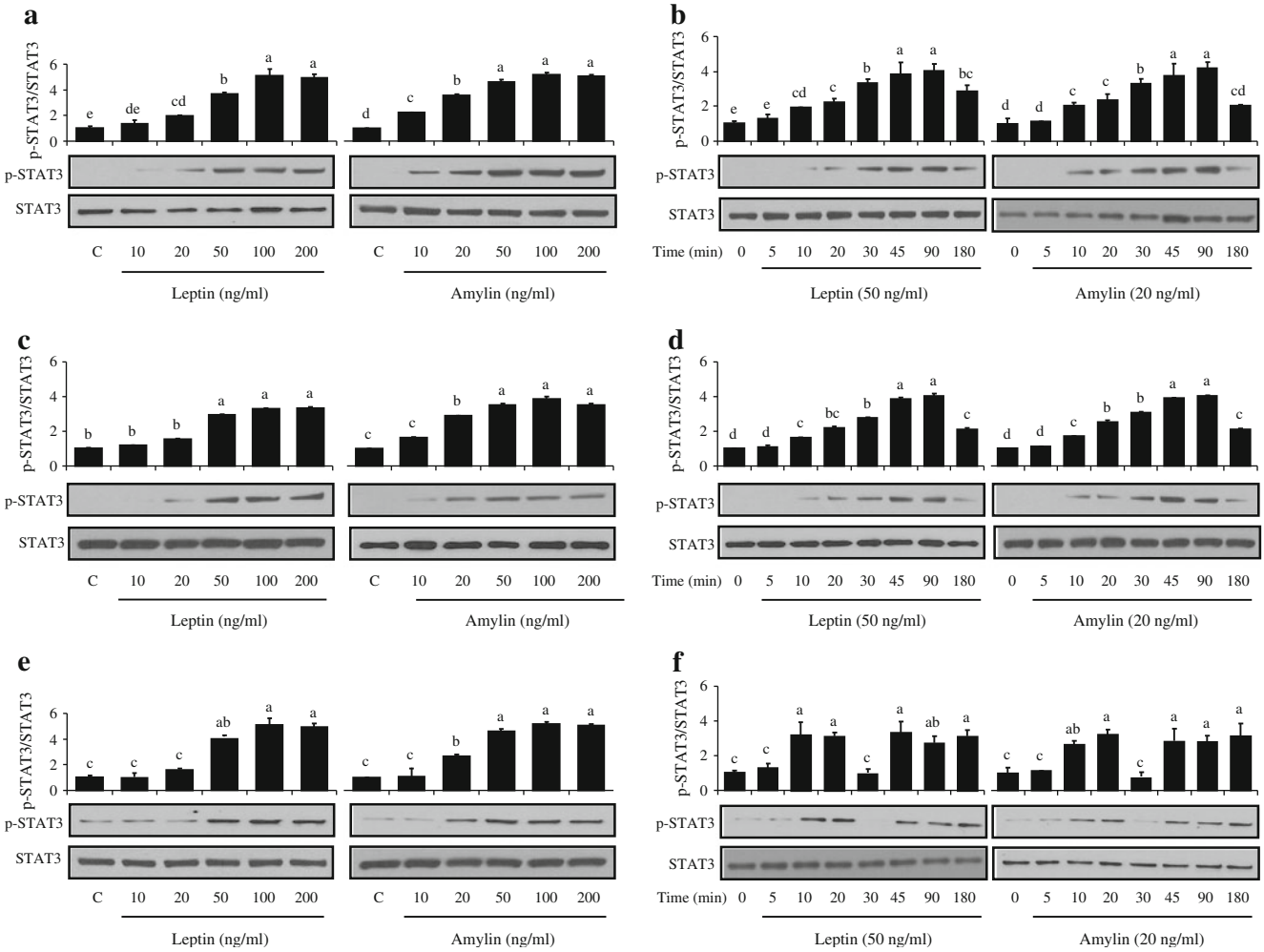

Fig. 1 Dose– and time–response curves in mouse GT1-7 hypothalamic (**a**, **b**), C_2C_{12} muscle (**c**, **d**) and AML12 liver (**e**, **f**) cell lines. The cell culture was performed as described (Methods, Cell culture); p-STAT3, phosphorylated STAT3. **a**, **c**, **e** The cells were treated for 45 min with leptin and/or amylin at the indicated concentrations. **b**, **d**, **f** The cells were treated at the indicated times with 50 ng/ml leptin and/or 20 ng/ml amylin. All density values for each protein band of interest are expressed as a fold increase. All data were analysed using one-way ANOVA followed by a post hoc test for multiple comparisons. Values are means ± SD; $n=3$; $p<0.05$ for any given superscript letter vs all others. C, control

compared with leptin or amylin administration alone. Importantly, we observed an additive, but not synergistic effect of amylin on leptin-mediated STAT3 (Fig. 2a–c), AMPK (Fig. 2a–c), ERK1/2 (Fig. 4a) and Akt (Fig. 4b) activation in all mouse cell lines.

Inhibition of STAT3 and AMPK signalling by ER stress in mouse GT1-7 hypothalamic, C_2C_{12} muscle and AML12 liver cell lines Stimulation of the cells with leptin, amylin and/or leptin + amylin led to a marked and significant increase in phosphorylation of STAT3 (Fig. a, c, e) and AMPK (Fig. 5b, d, f) compared with control in all cell lines. However, when challenged with the ER stress inducers tunicamycin and/or dithiothreitol, the activation of STAT3 and AMPK signalling by leptin + amylin was abolished. We also confirmed that tunicamycin and dithiothreitol increased the phosphorylation of eIF2 (positive control for ER stress) compared with control in all mouse cell lines (ESM Fig. 3).

The leptin-, but not the amylin-induced signalling pathway is mediated by IRS-1 in mouse GT1-7 hypothalamic, but not in C_2C_{12} muscle and AML12 liver cell lines To examine whether IRS activation is involved in leptin and/or amylin signalling, we directly measured the phosphorylation of IRS-1 and IRS-2 in response to leptin and amylin alone or in combination. This was done by analysis of phosphotyrosine levels of IRS-1 or IRS-2 immunoprecipitated with IRS-1 or IRS-2. We observed that treatment of C_2C_{12} muscle and AML12 liver cells with leptin and amylin alone or in combination did not activate IRS-1 (ESM Fig. 4a)

and IRS-2 (ESM Fig. 4b) compared with control. By contrast, leptin, but not amylin, activated IRS-1, but not IRS-2 in GT1-7 hypothalamic cell lines. Also, we observed that leptin activation of IRS-1 in GT1-7 hypothalamic cells is not further enhanced by co-administration of amylin.

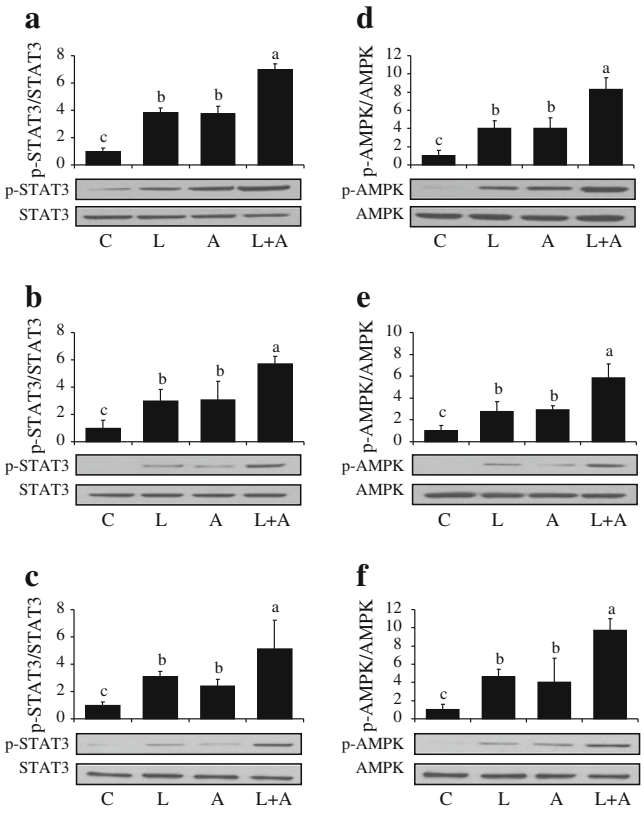

Fig. 2 Activation of STAT3 (**a–c**) and AMPK (**d–f**) signalling by administration of leptin and amylin alone or in combination in mouse GT1-7 hypothalamic, C_2C_{12} muscle and AML12 liver cell lines. The cell culture was performed as described (Methods, Cell culture); p-STAT3, phosphorylated STAT3; p-AMPK, phosphorylated AMPK. The cells were incubated for 45 min with 50 ng/ml leptin (L), 20 ng/ml amylin (A) and/or leptin + amylin (L + A); C, control. All density values for each protein band of interest are expressed as a fold increase. All data were analysed using one-way ANOVA followed by a post hoc test for multiple comparisons. Values are means ± SD; n=3; p<0.05 for any given superscript letter above bars vs all others. STAT3 (**a**) p=0.005 for C vs L, p=0.001 for C vs A and C vs L + A, p=1.000 for L vs A, p=0.004 for L vs L + A, p=0.001 for A vs L + A. **b** STAT3 (C_2C_{12}) p=0.004 for C vs L, p=0.005 for C vs A, p=0.001 for C vs L + A, p=1.000 for L vs A, p=0.005 for L vs L + A, p=0.001 for A vs L + A. **c** STAT3 (AML12) p=0.001 for C vs L, C vs A and C vs L + A, p=1.000 for L vs A, p=0.002 for L vs L + A, p=0.005 for A vs L + A. **d** AMPK (GT1-7) p=0.001 for C vs L, C vs A and C vs L + A, p=1.000 for L vs A, p=0.002 for L vs L + A, p=0.001 for A vs L + A. **e** AMPK (C_2C_{12}) p=0.001 for C vs L, p=0.005 for C vs A, p=0.002 for C vs L + A, p=1.000 for L vs A, p=0.005 for L vs L + A, p=0.001 for A vs L + A. **f** AMPK (AML12) p=0.005 for C vs L, p=0.002 for C vs A, p=0.005 for C vs L + A, p=1.000 for L vs A, p=0.005 for L vs L + A, p=0.001 for A vs L + A

Discussion

Amylin has been proposed to amplify leptin's effects, thereby synergistically affecting weight and fat loss in animals and humans [11, 12]. No previous study has investigated amylin and leptin signalling studies in hypothalamic, muscle and liver cells, nor has any prior study evaluated whether such signalling pathways are similar or overlapping, and whether administration of both hormones may have an additive or synergistic effect. To gain better insights into the effects of leptin and amylin in cells that are impossible or difficult to study directly in humans, such as hypothalamic, muscle or liver cells, we performed leptin and amylin signalling studies in mouse cell lines in vitro.

We first focused on STAT3, the prototype signalling molecule associated with the leptin receptor, which regulates food intake and body weight primarily in the hypothalamus [1, 18–20]. It has been proposed that co-administration of leptin and amylin in vivo in mice would result in a multiplicative effect on STAT3 phosphorylation in hypothalamus compared with treatment with each of the hormones separately [21]. Based on these results, we checked whether leptin could interact with amylin and whether co-treatment with amylin and leptin could further increase leptin-stimulated STAT3 signalling in mouse GT1-7 hypothalamic, C_2C_{12} muscle and AML12 liver cells. We observed that leptin and amylin increased STAT3 signalling and that the effect of these two hormones was similar in magnitude, peaking around the same time in mouse GT1-7 hypothalamic and C_2C_{12} muscle cell lines. By contrast, in mouse AML12 liver cell lines, leptin and amylin produced a biphasic response of STAT3 activity. Our data are consistent with previous rodent studies demonstrating that amylin-treated rats tend to have a greater number of cells positive for phosphorylated STAT3 than vehicle controls in the hypothalamus [21]. However, unlike previous in vivo studies, our in vitro data demonstrate only an additive effect. It also remains to be elucidated whether amylin may alter a third factor in vivo, which in turn may sensitise the hypothalamus to leptin in vivo, thus explaining the discrepancy between in vitro and in vivo findings.

No previous studies in animals or in humans have investigated STAT3 activation in liver and muscle. We recently demonstrated for the first time that amylin has direct effects on human adipose tissue and human peripheral blood mononuclear cells in vitro and ex vivo, and has additive, but not synergistic effects in leptin-stimulated signalling pathways [5]. In agreement with our previous study, we observed here an additive, but not synergistic, effect of amylin on leptin-stimulated STAT3 activation in mouse hypothalamic, muscle and liver cell lines. Importantly, leptin and amylin signalling pathways were saturable at a level of ~100 and ~50 to

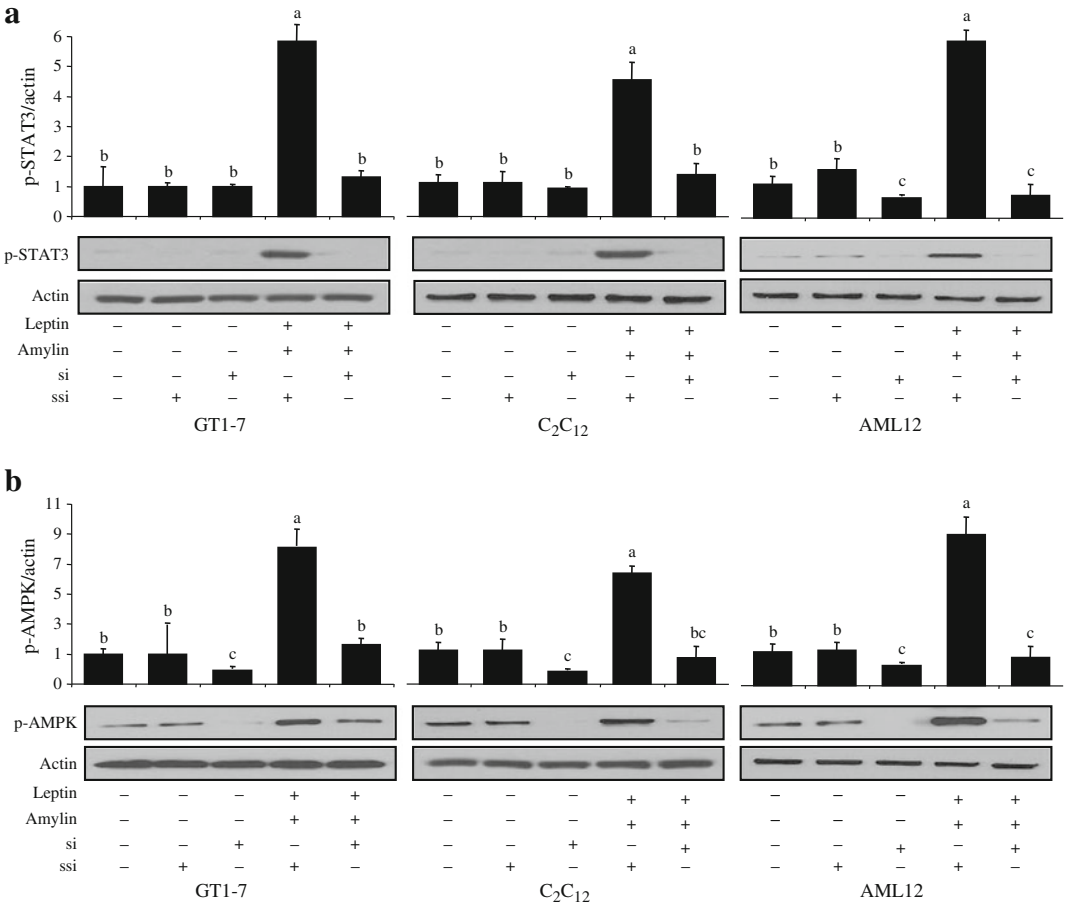

Fig. 3 Inhibition of (**a**) STAT3 and (**b**) AMPK signalling by administration of *Stat3* and *Ampk* siRNA in mouse cell lines as indicated. The cell culture was performed as described (Methods, Cell culture); p-STAT3, phosphorylated STAT3; p-AMPK, phosphorylated AMPK. The cells were transfected with *Stat3* and/or *Ampk* siRNA as described (Methods, Introduction of siRNA) and then stimulated for 45 min with 50 ng/ml leptin, 20 ng/ml amylin and/or leptin + amylin. All density values for each protein band of interest are expressed as a fold increase. All data were analysed using one-way ANOVA followed by a post hoc test for multiple comparisons. Values are means ± SD; $n=3$; $p<0.05$ for any given superscript letter vs all others

100 ng/ml, respectively, suggesting that no additional signalling effect can be observed at higher doses. Since STAT3 mediates the expression of various genes in response to cell stimuli and thus plays a key role in many cellular processes such as cell growth and apoptosis [4, 5, 20, 21], our data suggest that amylin-increased STAT3 signalling in leptin-stimulated mouse hypothalamic, muscle and liver cells may have important clinical implications.

We then focused on AMPK, another signalling pathway closely linked to leptin, which increases β-oxidation at the level of skeletal muscle and liver [8, 22]. It has been shown that leptin stimulates fatty acid oxidation and glucose transport in skeletal muscle and liver through activation of AMPK, suggesting that the metabolic effects of leptin are controlled by changes in the sub-cellular localisation of AMPK [23–26]. However, the question of whether amylin may activate AMPK to induce fatty acid oxidation in muscle and liver has not yet been studied; nor has amylin's action as a potential leptin sensitiser been elucidated. To address these questions, we checked whether leptin-activated AMPK signalling could be regulated by amylin, and whether a reduction of *Ampk* mRNA by siRNA alleviates leptin and amylin signalling in mouse hypothalamic, muscle and liver cell lines in vitro. We observed that leptin and amylin increased AMPK activation in mouse GT1-7 hypothalamic, C_2C_{12} muscle and AML12 liver cell lines. Under similar conditions, leptin- and/or amylin-induced increases of AMPK phosphorylation were prevented by administration of *Ampk* siRNA in all mouse cell lines. We did not observe any synergistic effect of amylin on leptin-stimulated AMPK activation in any of the cell lines studied, a finding consistent with our previous in vitro and ex vivo studies in humans [5]. Since AMPK is associated with fatty acid oxidation, which plays a key role in the pathophysiology of insulin resistance [15], leptin- and amylin-activated AMPK signalling in mouse hypothalamic, muscle and liver cells offers further support to the notion that these hormones, alone or in combination,

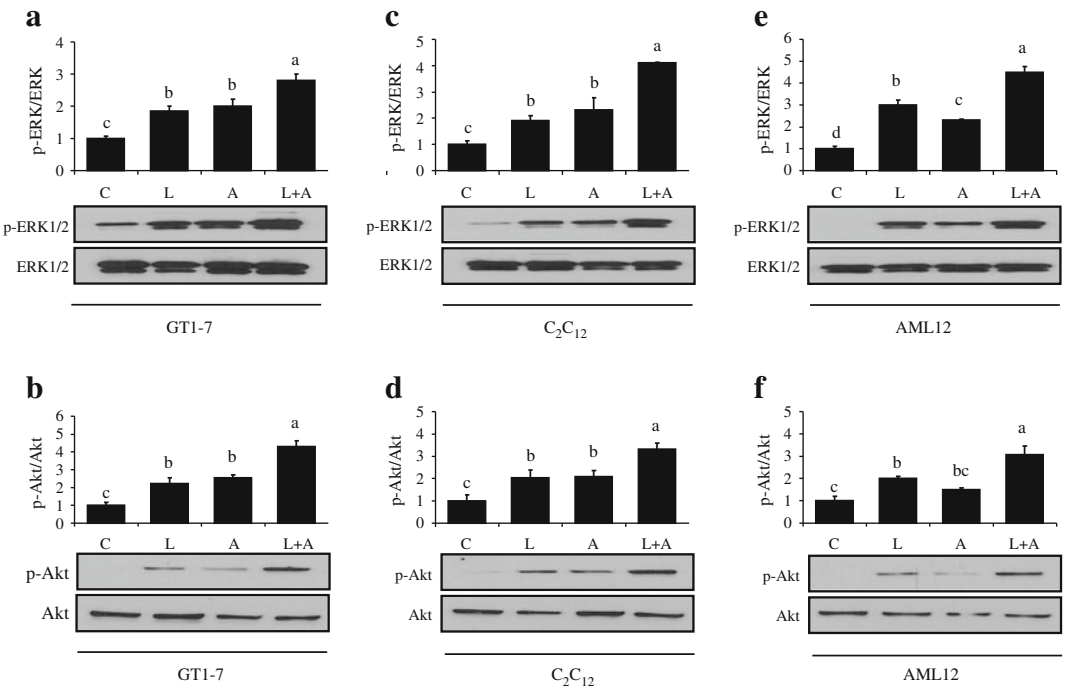

Fig. 4 Activation of ERK1/2 (**a**, **c**, **e**) and Akt (**b**, **d**, **f**) signalling by administration of leptin and amylin alone or in combination in mouse GT1-7 hypothalamic (**a**, **b**), C_2C_{12} muscle (**c**, **d**) and AML12 liver (**e**, **f**) cell lines. The cell culture was performed as described (Methods, Cell culture); p-ERK1/2, phosphorylated ERK1/2; p-Akt, phosphorylated Akt. The cells were incubated for 45 min with 50 ng/ml leptin (L), 20 ng/ml amylin (A) and/or leptin + amylin (L + A); C, control. All density values for each protein band of interest are expressed as a fold increase. All data were analysed using one-way ANOVA followed by a post hoc test for multiple comparisons. Values are means ± SD; $n=3$; $p<0.05$ for any given superscript letter vs all others. **a** ERK (GT1-7) $p=0.001$ for C vs L, C vs A and C vs L + A, $p=1.000$ for L vs A, $p=0.001$ for L vs L + A, $p=0.004$ for A vs L + A. **c** ERK (C_2C_{12}) $p=0.006$ for C vs L, $p=0.005$ for C vs A, $p=0.001$ for C vs L + A, $p=1.000$ for L vs A, $p=0.001$ for L vs L + A, $p=0.001$ for A vs L + A. **e** ERK (AML12) $p=0.001$ for C vs L, C vs A and C vs L + A, $p=1.000$ for L vs A, $p=0.001$ for L vs L + A and A vs L + A. **b** Akt (GT1-7) $p=0.005$ for C vs L, $p=0.001$ for C vs A and C vs L + A, $p=1.000$ for L vs A, $p=0.001$ for L vs L + A, and A vs L + A. **d** Akt (C_2C_{12}) $p=0.023$ for C vs L, $p=0.019$ for C vs A, $p=0.001$ for C vs L + A, $p=1.000$ for L vs A, $p=0.006$ for L vs A, $p=0.008$ for A vs L + A. **f** Akt (AML12) $p=0.004$ for C vs L and C vs A, $p=0.001$ for C vs L + A, $p=1.000$ for L vs A, $p=0.002$ for L vs L + A, $p=0.001$ for A vs L + A

could be viable for treating obesity and type 2 diabetes. Further in vivo studies in animals, and especially in humans, are needed to elucidate whether amylin administration may alter leptin signalling in vivo by altering a third intermediate factor that possibly could not be studied in vitro.

The ERK pathway is a chain of proteins in the cell that communicate a signal from a receptor on the surface to the DNA in the nucleus [27]. ERK is an additional pathway downstream of the leptin receptor [26, 28]; moreover, leptin has been shown to activate ERK1/2 in a time- and dose-dependent manner in several cultured mouse cells in vitro, as well as in rodent tissues in vivo [26]. The ERK pathway has been reported to mediate leptin's effects on human peripheral blood mononuclear cells in vitro, as well as on rat kidney and rat adipose tissue [8, 27]. Since ERK is the major kinase responsible for cell proliferation, differentiation and survival, and is also a significant downstream target for leptin's physiological effects [26], we further investigated whether amylin could activate ERK signalling in mouse hypothalamic, muscle and liver cell lines. We observed that amylin increased ERK activation in leptin-stimulated mouse hypothalamic, muscle and liver cell lines in vitro, this process occurring in a manner similar to the other signalling pathways discussed above.

Akt is a serine/threonine protein kinase that plays a key role in multiple cellular processes such as glucose metabolism, cell proliferation, apoptosis, transcription and cell migration [28]. It has been shown that leptin induces cell proliferation of hepatic stellate cells in vitro by activating the Akt signalling pathway [29] and stimulates proliferation and vascular endothelial growth factor secretion of endothelial cells in vitro [30]. We have previously demonstrated, for the first time, that leptin and amylin alone or in combination activate Akt signalling in human adipose tissues ex vivo and in human primary adipocytes and human peripheral blood mononuclear cells in vitro [5]. We demonstrate here that leptin and amylin alone or in combination activated Akt signalling in mouse hypothalamic, muscle and liver cell lines in vitro.

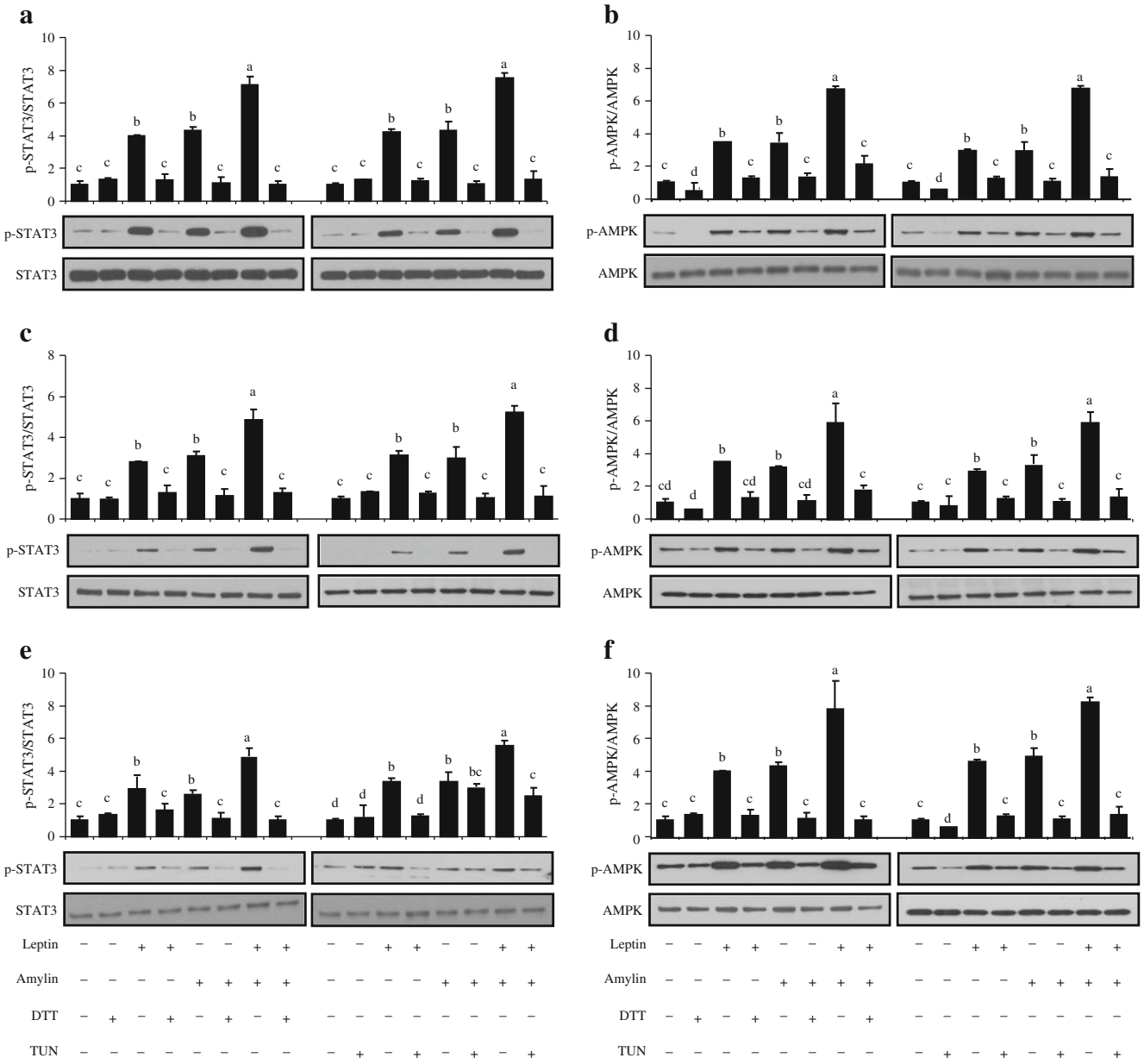

Fig. 5 Inhibition of STAT3 (**a**, **c**, **e**) and AMPK (**b**, **d**, **f**) signalling by ER stress in mouse GT1-7 hypothalamic (**a**, **b**), C_2C_{12} muscle (**c**, **d**) and AML12 liver (**e**, **f**) cell lines. The cell culture was performed as described (Methods, Cell culture); p-STAT3, phosphorylated STAT3; p-AMPK, phosphorylated AMPK. The cells were pre-incubated for 5 h with tunicamycin (3 μg/ml) (TUN) and/or dithiothreitol (1 mmol/l) (DTT), and then stimulated for 45 min with 50 ng/ml leptin, 20 ng/ml amylin and/or leptin + amylin. All density values for each protein band of interest are expressed as a fold increase. All data were analysed using one-way ANOVA followed by a post hoc test for multiple comparisons. Values are means ± SD; $n=3$; $p<0.05$ for any given superscript letter vs all others

We proceeded to assess whether amylin could function as a leptin sensitiser, as previously proposed [11], by studying whether it can ameliorate ER stress. Accumulating evidence suggests that stress signals lead to an accumulation of unfolded proteins, which cause ER stress [14]. This has recently been shown to affect leptin signalling and to play a role in the development of leptin resistance in the hypothalamus of rodents [13], suggesting that inhibition of STAT3 phosphorylation by ER stress is a potential mechanism underlying leptin resistance [14]. We have previously demonstrated that leptin- and amylin-activated STAT3 signalling are blocked by ER stress in human primary adipocytes in vitro [5]. Similarly, we observed here that leptin- and/or amylin-activated STAT3 signalling was blocked by ER stress inducers, i.e. tunicamycin and dithiothreitol, in mouse GT1-7 hypothalamic, C_2C_{12} muscle

and AML12 liver cell lines. These in vitro results indicate that amylin may not function as a leptin sensitiser to bypass ER stress-induced leptin resistance, but instead has an additive effect together with leptin. Similar studies need to be performed to determine whether the effect of amylin and leptin is similar in humans in vivo.

Although leptin has been demonstrated to directly affect the activation of signalling pathways that overlap with those of insulin in adipose tissue [8], no previous study has evaluated whether leptin and amylin signalling overlap with insulin signalling in mouse GT1-7 hypothalamic, C_2C_{12} muscle and AML12 liver cell lines. Hence, we directly studied the activation of IRS-1 and IRS-2 in response to leptin and amylin, alone or in combination, by measuring phosphotyrosine levels of IRS-1 or IRS-2 immunoprecipitated with IRS-1 or IRS-2. IRS-1 and IRS-2 are major transducers of insulin signalling in several cells/tissues, including fibroblasts, hepatocytes, muscle cells and adipose tissue [31–33]. It has been shown that insulin administration in rodents in vivo markedly activates IRS-1-associated phosphatidylinositol 3-kinases (PI3-kinases) in adipose tissue, liver and muscle [8]. Although leptin directly affects the activation of signalling pathways that overlap with those of insulin in adipose tissue [8], leptin did not activate IRS-1-associated PI3-kinase activity and no additive effects were observed between leptin and insulin [8]. In agreement with these results, we observed that treatment of C_2C_{12} muscle and AML12 liver cells with leptin and amylin alone or in combination did not induce increased phosphorylation of IRS-1 and IRS-2. By contrast, it has previously been shown that treatment with leptin activated Akt at 30 min and IRS-1 at 10 min in mouse muscle cell lines [17]. This previous study also showed that the effect of leptin on IRS-1 activation disappeared after 30 min, but still resulted in activation of Akt signalling. These results are consistent with IRS-1 activation being upstream of Akt phosphorylation. The exact timing of IRS-1 and Akt activation (and the relative sequence of their activation) needs to be elucidated in detail by future studies. We found that leptin, but not amylin activated IRS-1 in GT1-7 hypothalamic cell lines. These results suggest that the IRS-1 signalling pathway is involved in the potentiating action of leptin stimulation in GT1-7 hypothalamic cell lines. However, we were unable to identify any similar effects in cells from liver and muscle, i.e. peripheral tissues that may affect insulin resistance and metabolism.

It has been shown that leptin and amylin have a proliferative role in human and mouse cell lines [34–37]. In agreement with these previous studies, we observed that leptin- or amylin-activated cell proliferation was further increased by co-administration of both in all mouse cell lines studied here. Moreover, consistent with our signalling studies in mouse cell lines presented here, we also found that leptin and amylin had additive, but not synergistic, effects on cell proliferation activity. These results indicate that leptin and amylin may play a role in enhancing survival of mouse GT1-7 hypothalamic, C_2C_{12} muscle and AML12 liver cell lines.

In conclusion, despite minor differences in the timing of signalling activation, we did not observe major differences in the magnitude of STAT3, AMPK, ERK1/2 and/or Akt activation in response to leptin or amylin administration in the three mouse cell lines studied. We did, however, observe that leptin- and/or amylin-activated signalling pathways were abolished under ER stress conditions, suggesting that in all mouse cell lines studied amylin does not function as a leptin sensitiser to overcome ER stress-induced leptin resistance. Importantly, the leptin and amylin signalling pathways were saturable at a level of ~100 and ~50 to 100 ng/ml, respectively, which suggests that no additional signalling effect can be observed at higher doses than the above. Since in vivo leptin and amylin actions in humans may differ from those in mouse in vitro, studies of ex vivo and/or in vivo leptin and amylin signalling in humans are needed to confirm or refute the mouse in vitro signalling data. Although we studied the signalling pathways that are considered to be primary targets of leptin, on the basis of current evidence deriving mainly from rodent studies [8, 11, 12, 21, 26, 30, 36], we did not look at all possible signalling pathways. Thus, much more work needs to be done (additional tissues/cells, other signalling pathways) in the future. Despite these limitations, our initial data from mouse signalling studies suggest that leptin and amylin have additive, but not synergistic, effects on the activation of signalling pathways; they also provide novel insights into the role of STAT3, AMPK, ERK1/2 and Akt as probable mediators of the action of leptin and amylin in regulating energy homeostasis and insulin resistance/metabolism, either centrally in the hypothalamus [38, 39] and/or in the periphery [40–42].

Acknowledgements The Mantzoros Laboratory is supported by National Institute of Diabetes and Digestive and Kidney Diseases Grants 58785, 79929, 81913 and AG032030 and a VA Merit award. The Mantzoros Laboratory is also supported by a discretionary grant from Beth Israel Deaconess Medical Center.

Contribution statement HSM, JPC and CSM designed the study and analysed and interpreted data. HSM, JPC and CSM drafted and revised the article critically for important intellectual content. CSM conceived the study. All authors read and approved the final manuscript.

Duality of interest The authors state that there is no duality of interest associated with this manuscript.

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