original article

Diabetes, Obesity and Metabolism 14: 608–615, 2012.

ORIGINAL ARTICLE

Interactions of amylinergic and melanocortinergic systems in the control of food intake and body weight in rodents

J. D. Roth, L. D'Souza, P. S. Griffin, J. Athanacio, J. L. Trevaskis, R. Nazarbaghi, C. Jodka, J. Athanacio, J. Hoyt, B. Forood & D. G. Parkes

Amylin Pharmaceuticals, Inc., San Diego, CA, USA

Aims: Amylinergic and melanocortinergic systems have each been implicated in energy balance regulation. We examined the interactive effects of both systems using gene knockout and pharmacological approaches.

Methods: Acute food consumption was measured in overnight fasted male wild-type (WT) and melanocortin-4 receptor (MC-4R) deficient rats and in male and female WT and amylin knockout mice (AmyKO). Changes in food intake, body weight and composition in male WT and MC-4R deficient rats and in male diet-induced obese (DIO) rats. Pharmacological treatments included either rat amylin, murine leptin and/or the MC-4R agonist, Ac-R[CEH-dF-RWC]-amide.

Results: Amylin (10 μg/kg, IP) decreased food intake in WT but not in MC-4R deficient rats (30 and 60 min post-injection). Ac-R[CEH-dF-RWC]-amide (100 μg/kg, IP) suppressed food intake similarly in male WT and AmyKO, but was ineffective in female AmyKO. Amylin (50 μg/kg/day for 28 days) and leptin (125 μg/kg/day) synergistically reduced food intake and body weight in WT and MC-4R deficient rats to a similar extent. Amylin (100 μg/kg) combined with Ac-R[CEH-dF-RWC]-amide (100 μg/kg, IP) decreased acute food intake over 3 h to a greater extent than either agent alone in fasted mice. In DIO rats, additive anorexigenic, weight- and fat-lowering effects were observed over 12 days with the combination of rat amylin (50 μg/kg/day) and Ac-R[CEH-dF-RWC]-amide (2.3 mg/kg, SC injected daily).

Conclusions: Although amylin's acute anorexigenic effects are somewhat blunted in MC-4R deficiency and those of MC-4R agonism in amylin deficiency, these effects are surmountable with pharmacological administration lending therapeutic potential to combined amylin/melanocortin agonism for obesity.

Keywords: amylin, body weight obesity, combination, food intake, melanocortins

Date submitted 28 November 2011; date of first decision 22 December 2011; date of final acceptance 19 January 2012

Introduction

Among the peripheral signals implicated in the control of energy balance is amylin, a 37-amino acid peptide hormone produced in pancreatic β-cells and co-secreted with insulin in response to nutrient ingestion. In diet-induced obese (DIO) rats and overweight/obese humans, the weight-lowering effects of amylin agonism have been repeatedly showed, and mechanistic studies in rodents suggest that body weight is reduced in a fat-specific, lean mass-preserving manner [1–3]. Amylin agonism has also been shown to restore leptin responsiveness in preclinical models, as well as in obese humans [4–6].

An important downstream regulator of leptin is the hypothalamic melanocortin (MC) systems. Convergent lines of evidence underscore the importance of the MC subtype-4 receptor (MC-4R) as a key regulator of feeding behaviour. MC-4R knockout mice are severely obese [7], MC-4R antagonism stimulates food intake and weight gain [7,8] and mutations in the human MC-4R have been associated with obesity [9]. Peptidic and small molecule MC-4R agonists have received significant interest as potential antiobesity drugs [10,11].

To our knowledge the interaction of amylinergic and melanocortinergic systems has only been investigated to a limited extent. Amylin retained its acute anorexigenic effects in agouti mice (in which ectopic expression of the agouti-related protein (AgRP) antagonizes central MC-3Rs and MC-4Rs) and sustained administration of amylin, but not caloric restriction, modestly upregulated hypothalamic pro-opiomelanocortin (POMC) levels in DIO rats [3]. Our present studies further investigate the interactive effects of these two systems by: (i) exploring the antiobesity effects of amylin in an MC-4R-deficient state, and conversely, MC-4R agonism in an amylin-deficient state, (ii) establishing whether MC-4Rs are necessary for the synergistic action of amylin/leptin to reduce body weight, and (iii) evaluating the utility of amylin/MC-4R combination therapy in DIO rats.

Materials and Methods

Animals, Housing, Diet and Drug

All studies were approved by the Institutional Animal Care and Use Committee at Amylin Pharmaceuticals, in accordance

Correspondence to: Jonathan D. Roth, PhD, Amylin Pharmaceuticals, Inc., 9360 Towne Centre Drive, San Diego, CA 92121, USA.
E-mail: jonathan.roth@amylin.com

with Animal Welfare Act guidelines. Animals were housed individually in standard caging at 22 °C in a 12-h light, 12-h dark cycle.

Food intake studies were performed in normal female NIH Swiss mice (Harlan, Indianapolis, IN, USA). Studies using mice deficient for the amylin gene, hereafter referred to as amylin knockout (AmyKO) mice, were backcrossed at least six generations onto the C57Bl/6J strain (as described in Ref. [6]). The original AmyKO line was established on a mixed 129Ola/B6 background in which the amylin coding sequence in exon 3 of the amylin gene was replaced by a neomycin resistance cassette. AmyKO and WT littermate controls were genotyped at weaning (Taconic, Hudson, NY, USA). All mice were maintained *ad libitum* on standard chow (7012; Harlan Teklad, Madison, WI, USA).

Male MC-4R deficient rats ($Mc4r^{K314X/K314X}$) and littermate WT controls were obtained from Transposagen Biopharmaceuticals (Lexington, KY, USA). These rats carry an *N*-ethyl-*N*-nitrosourea-induced mutation that introduces a premature stop codon in the eighth helix of *Mc4r*, resulting in a full knockout phenotype [12]. WT and $Mc4r^{K314X/K314X}$ were maintained *ad libitum* on a moderately high-fat diet (32% kcal from fat; Research Diets D1226B, New Brunswick, NJ, USA).

DIO rat studies used male Sprague–Dawley rats from Charles River Laboratories (CRL: CD rats; Wilmington, MA, USA). DIO rats were also maintained *ad libitum* on the moderately high-fat diet (Research Diets D1226B) for approximately 8 weeks before and during treatment to induce a DIO state.

Murine leptin was recombinantly synthesized at Amylin Pharmaceuticals (San Diego, CA, USA) and was dissolved in sterile water for injection and infusion studies. Rat amylin was obtained from Peptisyntha (Torrance, CA, USA) and dissolved in 10% dimethyl sulfoxide (DMSO)/sterile water for acute injection studies and 50% DMSO/sterile water for minipump infusion studies. The MC-4R agonist Ac-R[CEH-dF-RWC]-amide was synthesized at Amylin Pharmaceuticals as previously described [13] and dissolved in saline before injection. This compound was selected from the series of MC analogues because it displayed potent binding affinity and functional activity for MC-4R (Ki = 0.4 nM; EC_{50} = 0.4 nM) versus MC-3R (Ki = 39.4 nM) [13].

Study 1: Acute Food Intake Study in $Mc4r^{K314X/K314X}$ Rats

Male WT and $Mc4r^{K314X/K314X}$ (n = 6/group) rats were fasted overnight and injected IP with either vehicle or rat amylin (10 μg/kg). Food was returned 30 min later and cumulative intake (corrected for spillage) was measured at 30, 60 and 120 min. The effects of amylin agonism were not assessed in female $Mc4r^{K314X/K314X}$ rats because of their limited commercial availability.

Study 2: Acute Food Intake Study in MC-4R-treated WT and AmyKO Mice

Male and female WT and AmyKO mice (n = 12/group) were fasted overnight and IP injected with either saline or the selective MC-4R agonist Ac-R[CEH-dF-RWC]-amide (100 μg/kg). Food was returned 30 min later and cumulative intake (corrected for spillage) was measured at 30, 60, 120, 180 and 240 min.

Study 3: Effects of Amylin and/or Leptin on 28-day Food Intake and Body Weight in WT and $Mc4r^{K314X/K314X}$ Rats

For chronic infusion studies we selected doses of amylin and leptin that reliably synergize to promote weight loss in lean and DIO rat models [5,6]. Each drug (or vehicle) was delivered by a separate surgically implanted subcutaneous (SC) osmotic minipump (Durect Corporation, Cupertino, CA, USA) containing either drug or vehicle (50% DMSO/sterile water for amylin, sterile water for leptin). Food intake (corrected for spillage) and body weight (expressed as percent vehicle corrected due to the differing starting body weights of these strains) were recorded on days 0, 1, 3, 7, 14, 21 and 28. At the start of the study body weights (in grams ± s.e.m.) of the treatment groups were: WT-vehicle, 520 ± 9; WT-amylin, 523 ± 12; WT-leptin, 524 ± 14; WT-amylin + leptin, 521 ± 13; $Mc4r^{K314X/K314X}$-vehicle, 568 ± 33; $Mc4r^{K314X/K314X}$ -amylin, 564 ± 20; $Mc4r^{K314X/K314X}$-leptin, 566 ± 9; $Mc4r^{K314X/K314X}$-amylin + leptin, 567 ± 4.

Study 4: Effects of the Amylin/MC-4R Combination on Food Intake in Overnight Fasted Mice

This study used group-housed (n = 3/cage) female NIH Swiss mice (24–30 g). Cages were randomized into treatment groups (n = 4 cages per peptide). All mice within a cage (n = 3 per cage) received the same treatment. Mice received an IP injection either peptide or vehicle, and food intake per cage was measured 30, 60, 120 and 180 min post-injection.

Study 5: Effects of Combined Amylin and MC-4R Agonism on Food Intake, Body Weight and Body Composition in DIO Rats

This study was designed to test the interactive effects of rat amylin and an MC-4R selective agonist on body weight and food intake. We selected a dose of amylin (50 μg/kg/day) that has reliably shown additive/synergistic effects on weight loss with a variety of test agents [14–16]. A pilot dose response study was conducted in rats to identify a dose of Ac-R[CEH-dF-RWC]-amide that would suppress food intake over 24 h. DIO rats were injected with either 0.46, 2.3 or 4.6 mg/kg and 24-h food intake was reduced by 20, 35 and 47%, respectively. Based on these findings, we selected a dose of 2.3 mg/kg for repeated administration. All rats were implanted subcutaneously with an osmotic minipump delivering either vehicle or amylin. Each rat also received a single daily SC injection of the MC-4R agonist just before the dark cycle. DIO rats (mean body weight, 607 ± 27 g) were assigned to the following treatment groups: vehicle, amylin (50 μg/kg/day by infusion), Ac-R[CEH-dF-RWC]-amide (2.3 mg/kg/day, daily injection) or amylin (50 μg/kg/day) + Ac-R[CEH-dF-RWC]-amide (2.3 mg/kg, daily injection). Food intake (corrected for spillage) and body weight measurements were taken daily. Body

composition was assessed at baseline and termination using a nuclear magnetic resonance (NMR) instrument as previously described [3].

Statistical Analyses

Data were analysed using one- and two-way (e.g. genotype × drug; time as a repeated measure when needed) analysis of variance (ANOVA) with post-hoc comparisons where appropriate. Significance was assumed for $p < 0.05$. Graphs were generated using Prism 4 for Windows (Graphpad Software, San Diego, CA, USA). All data points are expressed as mean ± standard error of the mean.

Results

Study 1: Acute Food Intake Study in WT and Mc4r$^{K314X/K314X}$ Rats

The anorexigenic effects of amylin differed in WT and *Mc4r*$^{K314X/K314X}$ rats ($p < 0.05$ drug × genotype; figure 1A–C). Amylin significantly reduced food intake only in WT (at 30 and 60 min; $p < 0.05$ vs. WT vehicle controls) but not in *Mc4r*$^{K314X/K314X}$ rats.

Study 2: Acute Food Intake Study in MC-4R-treated WT and AmyKO Mice

Ac-R[CEH-dF-RWC]-amide exerted similar effects in male WT and AmyKO mice (figure 2A). Food intake was decreased at the 60, 120, 180 and 240 min time-points relative to vehicle controls ($p < 0.05$ effect of drug, no effect of strain). In WT females, food intake was significantly decreased relative to vehicle controls at 120, 180 and 240 min ($p < 0.05$ effect of drug; figure 2B). However, Ac-R[CEH-dF-RWC]-amide failed to suppress food intake in female AmyKO mice at any of the time points.

Study 3: Effects of Amylin and/or Leptin on 28-day Food Intake and Body Weight in WT and Mc4r$^{K314X/K314X}$ Rats

The failure of amylin to reduce acute food intake in a state of MC-4R deficiency led us to hypothesize that with repeated dosing, amylin's anorexigenic and weight lowering effects would be diminished, and furthermore, the synergy of amylin and leptin would be absent in *Mc4r*$^{K314X/K314X}$ rats. At the dose tested, monotherapy with leptin was ineffective at suppressing cumulative food intake in WT rats (figure 3A). Amylin decreased food intake by 15% ($p < 0.05$ relative to vehicle controls on days 14, 21 and 28; figure 3A). Amylin/leptin decreased food intake by 22% ($p < 0.05$ relative to vehicle controls on days 10, 14, 21 and 28; figure 3A), but intake was not significantly different relative to amylin alone. For body weight, leptin was ineffective as a monotherapy and amylin reduced body weight by ~5% ($p < 0.05$ relative to vehicle controls on days 7, 14, 21 and 28; figure 3B). The addition of an ineffective dose of leptin enhanced the weight-lowering effects of amylin approximately twofold (to 10% by day 28; $p < 0.05$ relative to amylin monotherapy on days 14, 21 and 28; figure 3B).

In *Mc4r*$^{K314X/K314X}$ rats, leptin was ineffective at reducing cumulative food intake. Amylin decreased food intake by ~19% ($p < 0.05$ vs. vehicle controls on days 10, 14, 21 and 28) and amylin/leptin further decreased food intake by ~29% ($p < 0.05$ vs. amylin alone on day 28 only; figure 3C). For body weight, leptin was ineffective as a monotherapy and amylin reduced body weight by ~6.6% ($p < 0.05$ relative to vehicle controls on days 10, 14, 21 and 28; figure 3D). The addition of an ineffective dose of leptin enhanced the weight-lowering effects of amylin ~2.1-fold (to 14% by day 28; $p < 0.05$ relative to amylin monotherapy on days 21 and 28).

Study 4: Effects of the Amylin/MC-4R Combination on Food Intake in Overnight Fasted Mice

This study explored whether combined amylin and MC-4R agonism could exert co-operative anorexigenic effects. In line with its short half-life and previous reports [16,18], amylin pre-treatment significantly decreased food intake at 30 and 60 min ($p < 0.05$ vs. vehicle controls) but then food intake returned to the level observed in vehicle controls. The effects of Ac-R[CEH-dF-RWC]-amide were longer-lasting with significant suppression still evident at 180 min post-injection ($p < 0.05$ vs. vehicle controls 30, 60, 120 and 180 min). The combination of amylin and Ac-R[CEH-dF-RWC]-amide exerted a greater suppression relative to either agent alone at all time points

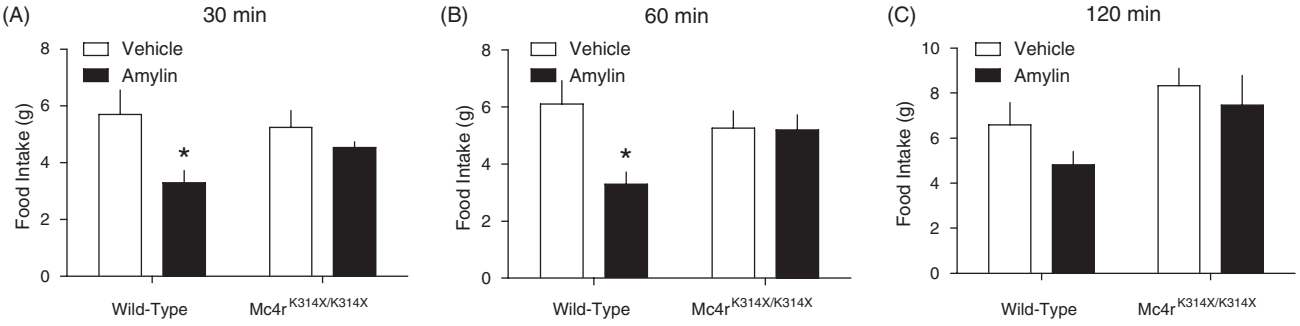

Figure 1. Anorexigenic effects of amylin (10 µg/kg, IP) in WT and *Mc4r*$^{K314X/K314X}$ rats at 30 (A), 60 (B) and 120 (C) min after injection. There was a significant drug × genotype interaction; *$p < 0.05$ indicates effect of drug in WT controls only.

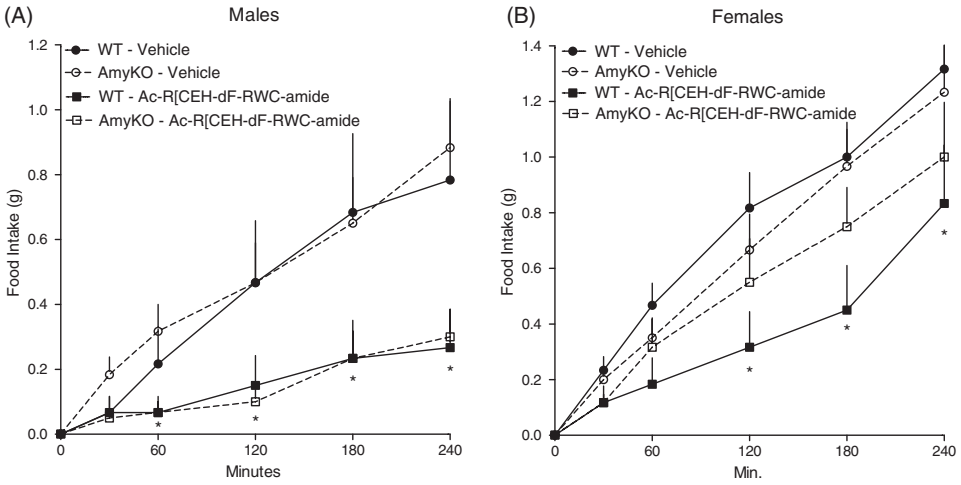

Figure 2. Anorexigenic effects of Ac-R[CEH-dF-RWC]-amide (100 μg/kg, IP) in male (A) and female (B) WT (solid lines) and AmyKO (dashed lines) mice. In male mice, there was no significant effect of drug × genotype; *$p < 0.05$ effect of drug. In females, a drug × genotype interaction was observed; *$p < 0.05$ indicates effect of drug in WT controls only.

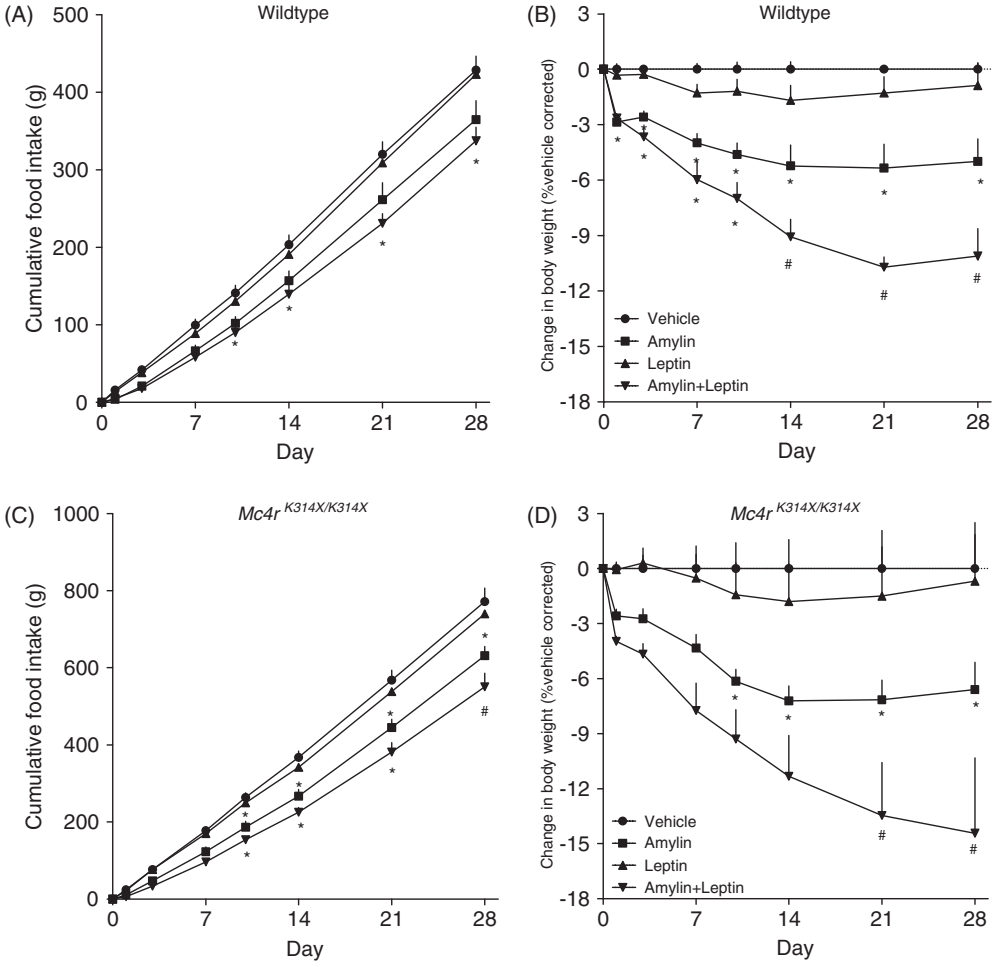

Figure 3. Effects of 28-day infusion of amylin (50 μg/kg/day; filled squares), leptin (125 μg/kg/day; filled triangles) or the combination of amylin (50 μg/kg/day) + leptin (125 μg/kg/day; inverted filled triangles) on cumulative food intake in grams (top left and right panels) and vehicle-corrected change in percent body weight (bottom left and right panels) in WT (A, B) and $Mc4r^{K314X/K314X}$ rats (C, D). No significant effect of drug × genotype. *$p < 0.05$ effect of drug in WT and $Mc4r^{K314X/K314X}$ rats.

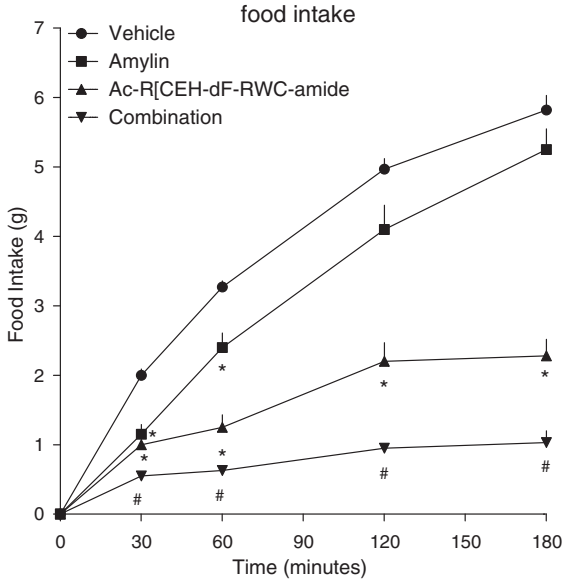

Figure 4. Anorexigenic effects of amylin (100 μg/kg, IP; filled squares), Ac-R[CEH-dF-RWC]-amide (1 mg/kg, IP; filled triangles) and amylin+ Ac-R[CEH-dF-RWC]-amide (filled inverted triangles) in female mice. *$p < 0.05$ monotherapy different from vehicle; #$p < 0.05$ combination different from monotherapy.

measured ($p < 0.05$ vs. amylin or Ac-R[CEH-dF-RWC]-amide; figure 4).

Study 5: Effects of Combined Amylin and MC-4R Agonism on Food Intake, Body Weight and Body Composition in DIO Rats

This study explored whether the co-operative acute anorexigenic effects of amylin and MC-4R agonism observed in mice would translate into mathematically additive (or greater) anorexigenic, weight- and fat-lowering effects in DIO rats. Individually, amylin (50 μg/kg/day) and Ac-R[CEH-dF-RWC]-amide (2.3 mg/day) decreased food intake (by 35 and 36%, respectively; figure 5A) and body weight by (6.1% or 31.5 g and 6.8% or 35.5 g, respectively, after 12 day of treatment; $p < 0.05$ vs. vehicle controls for food intake and body weight on all days; figure 5B). The combination of amylin and Ac-R[CEH-dF-RWC]-amide decreased cumulative food intake by 62% ($p < 0.05$ vs. monotherapy from day 5 and on; figure 5A) and body weight by 14.4% or 81.4 g ($p < 0.05$ vs. monotherapy from day 3 and on; figure 5B). Monotherapy with amylin or Ac-R[CEH-dF-RWC]-amide resulted in significant fat loss (16.0 and 23.7 g respectively, $p < 0.05$ vs. vehicle; figure 5C) with no significant lean loss (figure 5D). The combination of amylin and Ac-R[CEH-dF-RWC]-amide resulted in even greater fat loss (56.7 g, $p < 0.05$ vs. either monotherapy) with some loss of lean ($p < 0.05$ vs. vehicle or Ac-R[CEH-dF-RWC]-amide; figure 5C, D).

Discussion

The present preclinical studies reveal novel features of amylin and MC pharmacology. First, our food intake studies suggest that the acute anorexigenic effects of exogenous amylin or MC-4R agonism are most evident when both endogenous systems are functional. Overnight fasted $Mc4r^{K314X/K314X}$ rats were non-responsive to an acute dose of amylin that was effective in WT rats, implying that amylin's acute food intake lowering effects may be MC-4R dependent. In line with these findings, responsiveness to the food intake lowering effects of the amylin analogue salmon calcitonin was diminished over a 24-h period in MC-4R-deficient mice relative to WT controls [19]. Amylin treatment (for 24 days), but not pair-feeding, was associated with increased hypothalamic POMC levels in DIO rats maintained on a 32% fat further supporting an interaction between these two systems [3]. However, other findings have not linked endogenous MC systems to the effects of amylin. In *ad libitum* fed Wistar rats maintained on an 18% fat diet, neither amylin nor salmon calcitonin affected POMC mRNA expression in the hypothalamic arcuate nucleus following an acute injection [20]. We have previously shown that chow-fed overnight fasted agouti and WT mice respond similarly to an acute dose of amylin [3]. With respect to these latter findings, one explanation may be that these are two different models of MC dysregulation; agouti mice are a model of ectopic endogenous MC-3/MC-4R antagonism (by agouti overexpression), whereas the $Mc4r^{K314X/K314X}$ rat is a receptor loss-of-function model. The methodological and procedural differences (e.g. species, diet composition, nutritive status) across these different studies may also explain these discrepant findings. Nevertheless, any deficiencies in acute amylin-responsiveness in our single-dose study (10 μg/kg, IP bolus) appear to be surmountable. Qualitatively similar weight- and food intake-lowering effects were evident in WT and $Mc4r^{K314X/K314X}$ rats following sustained infusion of a dose of amylin (50 μg/kg/day) previously shown in our DIO model to increase plasma amylin levels ~50-fold above endogenous [14].

With respect to acute MC responsiveness, we observed a sexually dimorphic response following the administration of Ac-R[CEH-dF-RWC]-amide to male and female AmyKO mice. While the acute anorexigenic effects of MC-4R agonism were preserved in male AmyKO rats, they were blunted in AmyKO female rats. Sexually dimorphic effects of MC agonists have not been noted in other models; to what extent these effects are unique to amylin-deficiency warrants further investigation. Future studies should also clarify whether amylin exerts sexually dimorphic effects in models of MC dysregulation (e.g. $Mc4r^{K314X/K314X}$ rats).

A synergistic *in vivo* interaction with amylin and leptin agonism has now been confirmed by several groups, although the underlying mechanisms have not been fully elucidated [4,21,22]. Given the interdependence of MC and leptin signalling, we hypothesized that MC-signalling may be an important contributor to amylin/leptin synergy. We tested this hypothesis in the $Mc4r^{K314X/K314X}$ rat model because mice do not appear to display as marked a weight-lowering synergy to the amylin/leptin combination as rats [12,23]. On a biochemical level, this mutation impaired membrane-binding and subsequent receptor non-functionality and *in vivo* these rats were found to be non-responsive to MC-4R ligands (e.g. the potent orexigen, $AgRP_{79-129}$ and the anorexigen melanotan-II,

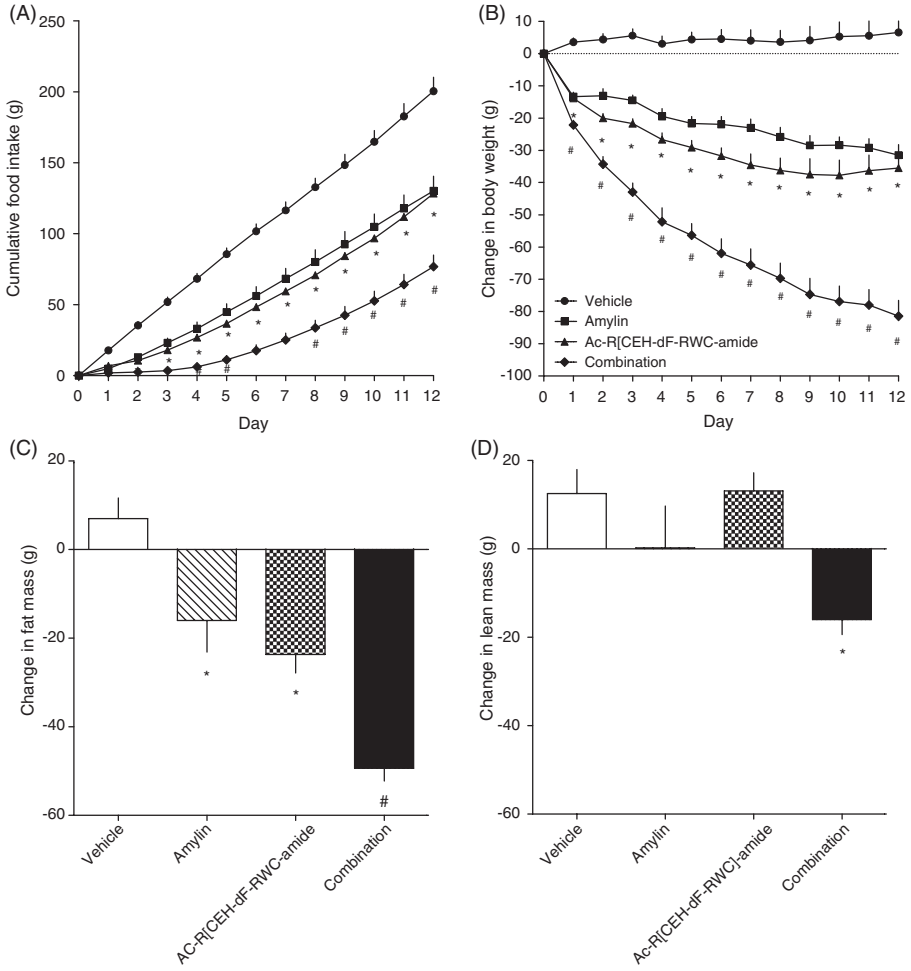

Figure 5. Effects of infusion of amylin (50 μg/kg/day) and/or injection of Ac-R[CEH-dF-RWC]-amide (2.3 mg/kg, IP once daily) on cumulative food intake (grams; upper left), body weight (grams; upper right), fat (grams; bottom left) and lean (grams; bottom right). $^{*}p < 0.05$ monotherapy different from vehicle; $^{\#}p < 0.05$ combination different from monotherapy.

MT-II). Contrary to our hypothesis, $Mc4r^{K314X/K314X}$ rats still exhibited greater than mathematically additive weight loss in response to sustained infusion of the combination of amylin/leptin. These findings suggest that intact MC-4R signalling is not necessary for the expression of amylin/leptin synergy. Although other MCR subtypes are presumably functional in this model (e.g. MC-3Rs that subtly contribute to food intake [24]), these observations point the possibility that amylin/leptin synergy may be mediated by MCR-independent mechanisms.

As part of our integrated neurohormonal therapeutic strategy for obesity, we have explored several anorexigenic and weight-lowering amylin-based combinations. Amylin agonism exerted additive effects with PYY(3-36) (in mice and rats [15]), phentermine and sibutramine (in rats [16] and humans [25]), synergistic effects with cholecystokinin (in mice [26] and rats [27]) and with leptin agonism (in rats [4,14] and humans [7]). Our combination studies point to the therapeutic utility of combined amylin and MC-4R agonism. To date, clinical results with monotherapy with MC agonists for obesity have been disappointing in terms of lack of efficacy. Although preclinical models have suggested that these agents cause weight and fat loss in obese rodents [28–30], clinical studies have failed to show meaningful weight loss [31] and have been linked to dose-limiting off target effects such as increases in blood pressure and erections [32]. Our preclinical findings suggest that amylin-based combinations with MC-4R agonism may be a novel therapeutic approach worthy of further consideration. Amylin co-administration enhanced the acute food intake lowering effects of Ac-R[CEH-dF-RWC]-amide in mice and with repeated co-administration durable, additive anorexigenic, weight- and fat-lowering effects in DIO rats. In preclinical models, MC agonism appears effective even under conditions of DIO leptin resistance [29,33]. If efficacious, combined amylin/MC agonism may be beneficial in cases where amylin agonism is unable to restore leptin responsiveness such as in highly leptin resistant states in the higher body mass index (BMI) ranges [21]. Multidose combination studies (e.g. employing response surface methodology or isobolographic analyses) can be used to identify whether optimal dose ratios exist for enhancing efficacy while remaining

subthreshold for tolerability related side effects as previously described [34].

Future mechanistic experiments should consider potential substrates for amylin/MC interactions including the dorsal hindbrain. Results from pharmacological, functional neuroanatomical and lesioning studies suggest that the energy regulatory effects of amylin rely upon direct activation of the area postrema (AP), which lacks a functional blood brain barrier [35]. Peripheral administration of MT-II activated AP neurons [36], and ^{125}I-MT-II binding has been detected within the AP [37]. The AP is situated adjacent to a population of MC-4R containing neurons in the dorsal motor nucleus of the vagus, which have been implicated in modulating food intake [38]. Amylin-activated AP neurons also project to the nucleus of the solitary tract (NTS) [35], which contains one of the two populations of POMC-synthesizing neurons (the other resides in the arcuate hypothalamus [39]). Manipulation of NTS POMC neurons impacts energy balance and ingestive behaviour (reviewed in Ref. [40]). Functional neuroanatomical and neuronal phenotyping studies can be used to explore whether the behavioural pharmacological interactions described herein arise via direct co-operative activation of AP neurons by amylin and MC-4R agonism and/or by amylin indirectly regulating components of the endogenous MC system (e.g. POMC and/or its conversion to α-melanocyte stimulating hormone, α-MSH).

In conclusion, amylin's acute anorexigenic effects are blunted in MC-4R deficiency and those of MC-4R agonism (in females) are attenuated in amylin deficiency. Nevertheless, these acute effects are surmountable with sustained pharmacological administration and co-operative effects are possible with amylin and MC-4R co-administration. Intact MC-4R signalling also does not appear to be required for amylin/leptin synergism. Given the preclinical-to-clinical translational fidelity of DIO rats for amylin-based combinations for weight loss, the present data suggest that amylin/MC-4R agonism is an antiobesity combination that warrants further investigation.

Acknowledgements

The authors wish to thank Jim Napora and the staff of the Comparative Medicine facility at Amylin Pharmaceuticals, Inc. for assistance with animal care.

Conflict of Interest

J.D.R. contributed to study design, data analyses and manuscript writing. L.D. and R.N. contributed to study design and synthetic chemistry. C.J. and J.L.T. contributed to study design and data analyses. P.G., J.A., J.A. and J.H. contributed to data collection and analyses. B.F. and D.G.P. contributed to study design and revision of the manuscript.

All authors are employees and hold equity in Amylin Pharmaceuticals, Inc.

References

1. Smith SR, Aronne LJ, Burns CM, Kesty NC, Halseth AE, Weyer C. Sustained weight loss following 12-month pramlintide treatment as an adjunct to lifestyle intervention in obesity. Diabetes Care 2008; **31**: 1816–1823.
2. Smith SR, Blundell JE, Burns C et al. Pramlintide treatment reduces 24-h caloric intake and meal sizes and improves control of eating in obese subjects: a 6-wk translational research study. Am J Physiol Endocrinol Metab 2007; **293**: E620–E627.
3. Roth JD, Hughes H, Kendall E, Baron AD, Anderson CM. Antiobesity effects of the beta-cell hormone amylin in diet-induced obese rats: effects on food intake, body weight, composition, energy expenditure, and gene expression. Endocrinology 2006; **147**: 5855–5864.
4. Roth JD, Roland BL, Cole RL et al. Leptin responsiveness restored by amylin agonism in diet-induced obesity: evidence from nonclinical and clinical studies. Proc Natl Acad Sci U S A 2008; **105**: 7257–7262.
5. Trevaskis JL, Lei C, Koda JE, Weyer C, Parkes DG, Roth JD. Interaction of leptin and amylin in the long-term maintenance of weight loss in diet-induced obese rats. Obesity 2010; **18**: 21–26.
6. Turek VF, Trevaskis JL, Levin BE et al. Mechanisms of amylin/leptin synergy in rodent models. Endocrinology 2010; **151**: 143–152.
7. Huszar D, Lynch CA, Fairchild-Huntress V et al. Targeted disruption of the melanocortin-4 receptor results in obesity in mice. Cell 1997; **88**: 131–141.
8. Raposinho PD, Castillo E, d'Alleves V, Broqua P, Pralong FP, Aubert ML. Chronic blockade of the melanocortin 4 receptor subtype leads to obesity independently of neuropeptide Y action, with no adverse effects on the gonadotropic and somatotropic axes. Endocrinology 2000; **141**: 4419–4427.
9. Farooqi IS, Yeo GS, Keogh JM et al. Dominant and recessive inheritance of morbid obesity associated with melanocortin 4 receptor deficiency. J Clin Invest 2000; **106**: 271–279.
10. Nargund RP, Strack AM, Fong TM. Melanocortin-4 receptor (MC4R) agonists for the treatment of obesity. J Med Chem 2006; **49**: 4035–4043.
11. He S, Ye Z, Dobbelaar PH et al. Discovery of a spiroindane based compound as a potent, selective, orally bioavailable melanocortin subtype-4 receptor agonist. Bioorg Med Chem Lett 2010; **20**: 2106–2110.
12. Mul JD, van Boxtel R, Bergen DJ et al. Melanocortin receptor 4 deficiency affects body weight regulation, grooming behavior, and substrate preference in the rat. Obesity 2011; doi:10.1038/oby.2011.81
13. Hsiung HM, Hertel J, Zhang XY et al. A novel and selective beta-melanocyte-stimulating hormone-derived peptide agonist for melanocortin 4 receptor potently decreased food intake and body weight gain in diet-induced obese rats. Endocrinology 2005; **146**: 5257–5266.
14. Trevaskis JL, Coffey T, Cole R et al. Amylin-mediated restoration of leptin responsiveness in diet-induced obesity: magnitude and mechanisms. Endocrinology 2008; **149**: 5679–5687.
15. Roth JD, Coffey T, Jodka CM et al. Combination therapy with amylin and peptide YY[3-36] in obese rodents: anorexigenic synergy and weight loss additivity. Endocrinology 2007; **148**: 6054–6061.
16. Roth JD, Trevaskis JL, Wilson J et al. Antiobesity effects of the beta-cell hormone amylin in combination with phentermine or sibutramine in diet-induced obese rats. Int J Obes 2008; **32**: 1201–1210.
17. Roth JD, Hughes H, Kendall E, Baron AD, Anderson CM. Anti-obesity effects of the β-cell hormone amylin in diet induced obese rats: effects on food intake, body weight, composition, energy expenditure and gene expression. Endocrinology 2006; **147**: 5855–5864.
18. Lutz TA, Geary N, Szabady MM, Del Prete E, Scharrer E. Amylin decreases meal size in rats. Physiol Behav 1995; **58**: 1197–1202.
19. Eiden S, Daniel C, Steinbrueck A, Schmidt I, Simon E. Salmon calcitonin - a potent inhibitor of food intake in states of impaired leptin signalling in laboratory rodents. J Physiol 2002; **541**: 1041–1048.
20. Barth SW, Riediger T, Lutz TA, Rechkemmer G. Differential effects of amylin and salmon calcitonin on neuropeptide gene expression in the lateral hypothalamic area and the arcuate nucleus of the rat. Neurosci Lett 2003; **341**: 131–134.

21. Osto M, Wielinga PY, Alder B, Walser N, Lutz TA. Modulation of the satiating effect of amylin by central ghrelin, leptin and insulin. Physiol Behav 2007; **91**: 566–572.
22. Seth R, Knight WD, Overton JM. Combined amylin-leptin treatment lowers blood pressure and adiposity in lean and obese rats. Int J Obes 2011; **35**: 1183–1192.
23. Trevaskis JL, Parkes DG, Roth JD. Insights into amylin-leptin synergy. Trends Endocrinol Metab 2010; **21**: 473–479.
24. Irani BG, Xiang Z, Yarandi HN et al. Implication of the melanocortin-3 receptor in the regulation of food intake. Eur J Pharmacol 2011; **660**: 80–87.
25. Aronne LJ, Halseth AE, Burns CM, Miller S, Shen LZ. Enhanced weight loss following coadministration of pramlintide with sibutramine or phentermine in a multicenter trial. Obesity 2010; **18**: 1739–1746.
26. Bhavsar S, Watkins J, Young A. Synergy between amylin and cholecystokinin for inhibition of food intake in mice. Physiol Behav 1998; **64**: 557–561.
27. Trevaskis JL, Turek VF, Griffin PS, Wittmer C, Parkes DG, Roth JD. Multihormonal weight loss combinations in diet-induced obese rats: therapeutic potential of cholecystokinin? Physiol Behav 2010; **100**: 187–195.
28. Yaswen L, Diehl N, Brennan MB, Hochgeschwender U. Obesity in the mouse model of pro-opiomelanocortin deficiency responds to peripheral melanocortin. Nat Med 1999; **5**: 1066–1070.
29. Pierroz DD, Ziotopoulou M, Ungsunan L, Moschos S, Flier JS, Mantzoros CS. Effects of acute and chronic administration of the melanocortin agonist MTII in mice with diet-induced obesity. Diabetes 2002; **51**: 1337–1345.
30. Tian X, Switzer AG, Derose SA et al. Discovery of orally bioavailable 1,3,4-trisubstituted 2-oxopiperazine-based melanocortin-4 receptor agonists as potential antiobesity agents. J Med Chem 2008; **51**: 6055–6066.
31. Krishna R, Gumbiner B, Stevens C et al. Potent and selective agonism of the melanocortin receptor 4 with MK-0493 does not induce weight loss in obese human subjects: energy intake predicts lack of weight loss efficacy. Clin Pharmacol Ther 2009; **86**: 659–666.
32. Greenfield JR. Melanocortin signalling and the regulation of blood pressure in human obesity. J Neuroendocrinol 2011; **23**: 186–193.
33. Bluher S, Ziotopoulou M, Bullen JW Jr et al. Responsiveness to peripherally administered melanocortins in lean and obese mice. Diabetes 2004; **53**: 82–90.
34. Roth JD, Trevaskis JL, Turek VF, Parkes DG. ''Weighing in'' on synergy: preclinical research on neurohormonal anti-obesity combinations. Brain Res 2010; **1350**: 86–94.
35. Potes CS, Lutz TA. Brainstem mechanisms of amylin-induced anorexia. Physiol Behav 2010; **100**: 511–518.
36. Rowland NE, Schaub JW, Robertson KL, Andreasen A, Haskell-Luevano C. Effect of MTII on food intake and brain c-Fos in melanocortin-3, melanocortin-4, and double MC3 and MC4 receptor knockout mice. Peptides 2010; **31**: 2314–2317.
37. Trivedi P, Jiang M, Tamvakopoulos CC et al. Exploring the site of anorectic action of peripherally administered synthetic melanocortin peptide MT-II in rats. Brain Res 2003; **977**: 221–230.
38. Grill HJ, Ginsberg AB, Seeley RJ, Kaplan JM. Brainstem application of melanocortin receptor ligands produces long-lasting effects on feeding and body weight. J Neurosci 1998; **18**: 10128–10135.
39. Bronstein DM, Schafer MK, Watson SJ, Akil H. Evidence that beta-endorphin is synthesized in cells in the nucleus tractus solitarius: detection of POMC mRNA. Brain Res 1992; **587**: 269–275.
40. Ellacott KL, Cone RD. The central melanocortin system and the integration of short- and long-term regulators of energy homeostasis. Recent Prog Horm Res 2004; **59**: 395–408.