Double deletion of orexigenic neuropeptide Y and dynorphin results in paradoxical obesity in mice

Amy D. Nguyen [a], Katy Slack [a], Christoph Schwarzer [b], Nicola J. Lee [a], Dana Boey [a], Laurence Macia [a], Ernie Yulyaningsih [a], Ronaldo F. Enriquez [c], Lei Zhang [a], Shu Lin [a], Yan-Chuan Shi [a], Paul A. Baldock [b], Herbert Herzog [a,d], Amanda Sainsbury [a,e,f]

[a] Neuroscience Research Program, Garvan Institute of Medical Research, 384 Victoria Street, Darlinghurst, Sydney, NSW 2010, Australia [b] Department of Pharmacology, Medical University Innsbruck, 6020 Innsbruck, Austria [c] Bone and Mineral Research Program, Garvan Institute of Medical Research, 384 Victoria Street, Darlinghurst, Sydney, NSW 2010, Australia [d] Faculty of Medicine, University of NSW, Kensington, Sydney, NSW 2052, Australia [e] School of Medical Sciences, University of NSW, Kensington, Sydney, NSW 2052, Australia [f] The Boden Institute of Obesity, Nutrition, Exercise & Eating Disorders, Sydney Medical School, The University of Sydney, Sydney, NSW 2006, Australia

ABSTRACT

Objective
Orexigenic neuropeptide Y (NPY) and dynorphin (DYN) regulate energy homeostasis. Single NPY or dynorphin deletion reduces food intake or increases fat loss. Future developments of obesity therapeutics involve targeting multiple pathways. We hypothesised that NPY and dynorphin regulate energy homeostasis independently, thus double NPY and dynorphin ablation would result in greater weight and/or fat loss than the absence of NPY or dynorphin alone.
Design and methods
We generated single and double NPY and dynorphin knockout mice (NPYΔ, DYNΔ, NPYDYNΔ) and compared body weight, adiposity, feeding behaviour, glucose homeostasis and brown adipose tissue uncoupling protein-1 (UCP-1) expression to wildtype counterparts.
Results
Body weight and adiposity were significantly increased in NPYDYNΔ, but not in NPYΔ or DYNΔ. This was not due to increased food intake or altered UCP-1 expression, which were not significantly altered in double knockouts. NPYDYNΔ mice demonstrated increased body weight loss after a 24-h fast, with no effect on serum glucose levels after glucose injection.
Conclusions
Contrary to the predicted phenotype delineated from single knockouts, double NPY and dynorphin deletion resulted in heavier mice, with increased adiposity, despite no significant changes in food intake or UCP-1 activity. This indicates that combining long-term opioid antagonism with blockade of NPY-ergic systems may not produce anti-obesity effects.

1. Introduction

The recent decision by the US Food and Drug Authority (FDA) to delay approval of the new anti-obesity drug, *Contrave* (Orexigen, 2011), has once again reignited the debate of whether the effectiveness of weight-loss drugs outweighs their side effects. *Contrave*, developed by Orexigen, is a fixed-dose combination of naltrexone, a non-selective opioid receptor antagonist, and bupropion, a selective dopamine reuptake inhibitor (Orexigen, 2011). Due to its cardiovascular effects (Orexigen, 2011), the FDA has sought extensive safety data prior to its approval as an oral obesity treatment. Overweight and obesity currently affect billions of people worldwide, particularly those in developed nations, and are now becoming increasingly prevalent in developing countries also (WHO, 2013). As the rates of this epidemic are rising alarmingly (WHO, 2013), with lifestyle interventions having low success rates (Mann et al., 2007) and surgery being the only effective treatment (Picot et al., 2009), albeit with side effects and not being suitable for everyone, the search for an elusive drug cure is rife.

A large majority of people with a body mass index in the overweight or obese range find that maintaining a reduced body weight in the long-term is a large, compounding hurdle in the quest for a healthy body weight. Up to 70% of those that lose weight using lifestyle

interventions alone regain the weight within 4 years (Sumithran and Proietto, 2013 and Wing and Phelan, 2005). This is due – at least in part – to weight loss activating adaptive responses that stimulate appetite and reduce energy expenditure, as recently reviewed (Sainsbury and Zhang, 2012). These adaptive responses to energy restriction are seemingly mediated by numerous hypothalamic peptides (Sainsbury and Zhang, 2010 and Sainsbury and Zhang, 2012), notably neuropeptide Y (NPY) (Stephens et al., 1995) and possibly also the opioid peptides, dynorphins (Kalra and Kalra, 1996 and Sainsbury et al., 2007), with the aforementioned drug, *Contrave*, utilising opioid receptor antagonism as one of its mechanisms of action.

Negative energy balance, such as during dieting and other lifestyle-based weight loss, leads to decreased circulating leptin levels (Belza et al., 2009) and – at least in rodents – subsequently increased hypothalamic NPY expression (Stephens et al., 1995). Increased central NPY-ergic tonus has been shown to result in increased appetite (Clark et al., 1984) and reduced physical activity (Heilig et al., 1989). These responses are associated with significant decreases in total energy expenditure and body temperature (Hwa et al., 1999), along with increases in food efficiency and fat accretion (Stanley et al., 1986). These effects of NPY contribute to obesity in rodents when leptin action is permanently reduced (Wong et al., 2013), and conceivably also contribute to body weight regain in people after lifestyle-based weight loss interventions (Sainsbury and Zhang, 2010 and Sainsbury and Zhang, 2012). Therefore, it would follow on that blocking the effects of increased hypothalamic NPY-ergic tonus, thus blocking these adaptive responses to energy restriction, could increase the effectiveness of weight loss interventions.

In addition to the NPY system, the three peptide families of the endogenous opioid system – endorphins, enkephalins and dynorphins, which preferentially act on mu (μ), delta (δ) and kappa (κ) opioid receptors respectively – are implicated in feeding and body weight regulation (Cooper, 1980, Grandison and Guidotti, 1977 and Thornhill et al., 1976). Of these endogenous opioid systems, κ opioid receptors have been particularly implicated in these processes (Arjune and Bodnar, 1990, Hamilton and Bozarth, 1988 and Levine et al., 1990). Specific blockade of κ-opioid receptors significantly reduces fasting-induced hyperphagia in rats (Lambert et al., 1993), as well as food intake and body weight in obese rodent models (Cole et al., 1995 and Jarosz and Metzger, 2002). Further evidence for a role of κ-opioid receptors in the regulation of energy balance is the observation that dynorphin knockout mice have significantly less white adipose tissue mass and lose more weight during a 24-h fast than wildtype mice (Sainsbury et al., 2007).

Evidence suggests that endogenous opioids may contribute to the energy-conserving, appetite promoting effects of negative energy balance, and that they do so via at least partially distinct and additive pathways to those activated by NPY (Cooper, 1980, Lambert et al., 1993 and Sainsbury et al., 2007). NPY and pre-prodynorphin mRNA and protein are co-localised in regions of the hypothalamus involved in energy homeostasis regulation, notably the arcuate nucleus (Lin et al., 2006). After a 24-h fast, there are increases in immunoreactivity levels of both NPY (in arcuate and paraventricular nuclei) and dynorphin (in the overall hypothalamus) (Przewlocki et al., 1983 and Sahu et al., 1988), suggesting potentially similar actions. Notably, double intracerebroventricular administration of a NPY antibody and κ-opioid receptor antagonist, norbinaltorphimine, resulted in additive inhibition of hyperphagia, greater than responses seen in single NPY or κ-opioid receptor system disruption (Lambert et al., 1993). This evidence provides support for the possibility that the NPY and dynorphin systems function at least partially independently of one another in the control of energy homeostasis.

Based on these findings of possible independent and hence additive actions of NPY and dynorphin in the regulation of energy balance, and with a view towards future development of pharmacological obesity treatments that target dual pathways, we hypothesised that double ablation of both NPY and dynorphin function would result in greater weight and/or fat loss than the absence of either NPY or dynorphin alone. In this work, we specifically tested this hypothesis using a double NPY and dynorphin knockout mouse model (NPYDYNΔ), with subsequent analysis of body weight, adiposity, food intake, brown adipocyte thermogenesis marker expression, as well as blood glucose response to glucose injection.

2. Methods and procedures

2.1. Ethics statement and animal care
All research and animal care procedures were approved by the Garvan Institute/St Vincent's Hospital Animal Ethics Committee and in agreement with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. All mice were group housed, unless otherwise stated, under conditions of controlled temperature (22 °C) and illumination (12 h light–dark cycle, lights on at 7:00 h) with *ad libitum* access to water and standard chow (8% calories from fat, 21% calories from protein, 71% calories from carbohydrates and 2.6 kcalories g^{-1}; Gordon's Specialty Stock Feeds, Yanderra, New South Wales, Australia).

2.2. Generation of knockout mice
All knockout and wildtype (WT) mice were on the same mixed C57BL/6–129/SvJ background. Single NPY and dynorphin knockout mice (NPYΔ and DYNΔ) were generated as previously published in (Karl et al., 2008) and (Loacker et al., 2007), respectively. Dynorphin knockout leads to deletion of the pre-prodynorphin gene, the common precursor of all five dynorphin peptides. Generation of double NPYDYN knockout mice (NPYDYNΔ) was accomplished through crossing of the single NPY and dynorphin knockout mice lines.

2.3. Body weight monitoring
After weaning, animals were weighed once a week at the same time of day throughout the duration of the experiment.

2.4. Tissue collection
At 13–14 weeks of age, mice were culled between 13:00 and 17:00 h by cervical dislocation followed by decapitation. The interscapular brown adipose tissue (BAT) as well as white adipose tissue (WAT) depots (right inguinal, right epididymal or periovarian (gonadal), mesenteric and right retroperitoneal) were removed and weighed. The weights of these WAT depots were presented individually and also summed together and expressed as total WAT weight, expressed in absolute weight as well as normalised as a percent of body weight. BAT samples were stored at −80 °C until subsequent analysis as described below.

2.5. Western blotting
BAT samples taken from male animals were homogenised in RIPA buffer (25 mM TrisNHCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) supplemented with Complete Protease Inhibitor Cocktail Tablets (Complete Mini, Roche Diagnostic, Mannheim, Germany). After centrifugation, clear lysates were collected and protein concentrations were measured by a microplate spectrophotometer (Spectramax Plus384, Molecular Devices Inc, Silicon Valley, California, USA) using a reagent from Biorad (Biorad, Gladesville, New South Wales, Australia). Equal amounts of tissue lysates (20 µg protein) were resolved by SDS–PAGE and immunoblotted with antibodies against uncoupling protein-1 (UCP-1) (Alpha Diagnostic International Inc, San Antonio, Texas, USA) and peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC1-α) (Calbiochem, Merck Pty Ltd, Kilsyth, Victoria, Australia). Immunolabelled bands were then quantified using densitometry.

2.6. Feeding studies
At 10–11 weeks of age, mice were transferred from group housing with soft bedding to individual cages with paper towel bedding and acclimatised to these new conditions for two to four nights. Food intake was measured at 11–12 weeks of age both in the fed state (spontaneous food intake), as well as in response to 24-h fasting. Spontaneous food intake was determined over a 24-h period, and 24-h fasting-induced food intake was measured at 24, 48 and 72 h after reintroduction of food. Body weight was tracked for the duration of the feeding studies. Actual food intake was calculated as the weight of food taken from the hopper minus the weight of food spillage in the cage. Mice were placed back onto soft bedding after completion of feeding studies.

2.7. Glucose metabolism
At 12–13 weeks of age, knockout and wildtype mice underwent a glucose tolerance test. Briefly, mice were fasted for 16–24 h before intraperitoneal injection between 13:00 and 15:00 h of a 10% d-glucose solution (1.0 g/kg) (Astra Zeneca, North Ryde, New South Wales, Australia). Blood samples were obtained from the tail tip at 0, 15, 30, 60 and 90 min after

glucose injection. Serum was stored at −20 °C for subsequent analysis of glucose using a glucose oxidase kit (Trace Scientific, Clayton, Victoria, Australia). Integrated areas under the resultant glucose response curves (AUC) were calculated (without subtracting baseline values) between $t = 0$ and $t = 90$ min after glucose injection, and are expressed in arbitrary units.

2.8. Statistical analyses

All data are expressed as means ± SEM. Differences among groups of mice were assessed by ANOVA (for BAT weight, total WAT weight, BAT UCP-1 and PGC1-α expression, spontaneous food intake, body weight lost after a 24-h fast, area under the curve for plasma glucose after intraperitoneal glucose injection) or repeated measures ANOVA (for body weight, individual WAT depot weights, 24-h fasting-induced feeding, body weight changes over 72 h, serum glucose curves following intraperitoneal glucose injection), with Fisher's post hoc tests where appropriate. Statistical analyses were performed with SPSS for Mac OS X version 16.0.1 (SPSS Inc, Chicago, Illinois, USA). Statistical significance was defined as $P < 0.05$.

3. Results

3.1. Body weight is increased after double NPY and dynorphin ablation

Measuring the body weight of wildtype (WT), NPYΔ, DYNΔ and NPYDYNΔ mice from 5 to 13 weeks of age revealed that both male and female NPYDYNΔ mice, as well as female DYNΔ mice, were significantly heavier than their wildtype counterparts (Fig. 1). Importantly, male but not female NPYDYNΔ mice were already heavier than wildtype mice at 5 weeks of age (Fig. 1).

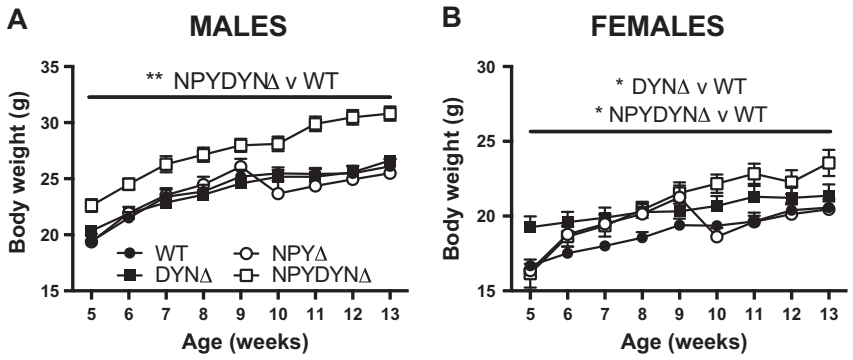

Fig. 1. Increased body weight in double NPYDYNΔ mice. Body weight of male (A) and female (B) wildtype (WT), NPYΔ, DYNΔ and NPYDYNΔ mice were measured weekly. Plotted values are means ± SEM of over 5 mice per group. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to WT mice.

3.2. Increased body weight in NPYDYNΔ mice is due to increased adiposity

At the conclusion of the experiment at 13–14 weeks of age, individual white adipose tissue depots were dissected and weighed to determine the degree of adiposity. Double ablation of NPY and dynorphin resulted in increased adiposity in both male and female mice, reflected by increased weight of the white adipose tissues, expressed either as absolute weight (Fig. 2A, B) or as a percentage of body weight (Fig. 2C, D). In addition to increased white adipose tissue mass, absolute and relative brown adipose tissue mass was significantly increased in male (Fig. 2A, C), but not female (Fig. 2B, D) NPYDYNΔ mice. In results broadly consistent with our previous observation of reduced fat mass in DYNΔ mice (Sainsbury et al., 2007), our current cohort of DYNΔ mice also showed reductions in white adipose tissue depot weights relative to wildtype values, significantly so in females for the total weight of these depots (Fig. 2).

3.3. Increased body weight gain and adiposity seen in NPYDYNΔ mice is not due to increased food intake

To determine whether the significant increases in body weight and adiposity of double knockout mice were due to an increase in energy intake, food intake was measured both

under spontaneous (Fig. 3) and fasting-induced conditions (Fig. 4). Under spontaneous conditions, there was no significant difference amongst genotypes with respect to food intake, except in female NPYΔ mice, which ate significantly more than their wildtype counterparts when intake was expressed in absolute terms (Fig. 3B), but not significantly so when normalised to body weight (Fig. 3D). In the post-fasting condition, food intake of double NPYDYNΔ mice was not significantly different from that of other groups, either when expressed as absolute intake or normalised to body weight (Fig. 4A–D). Interestingly, male DYNΔ mice ate significantly less than wildtype mice up to 72 h post-fast, when food intake was expressed as absolute intake (Fig. 4A), but not when expressed as a percent of body weight (Fig. 4C). No such hypophagia was observed in female DYNΔ mice relative to wildtype counterparts, consistent with our previous observation of no effect of dynorphin ablation on food intake (Sainsbury et al., 2007).

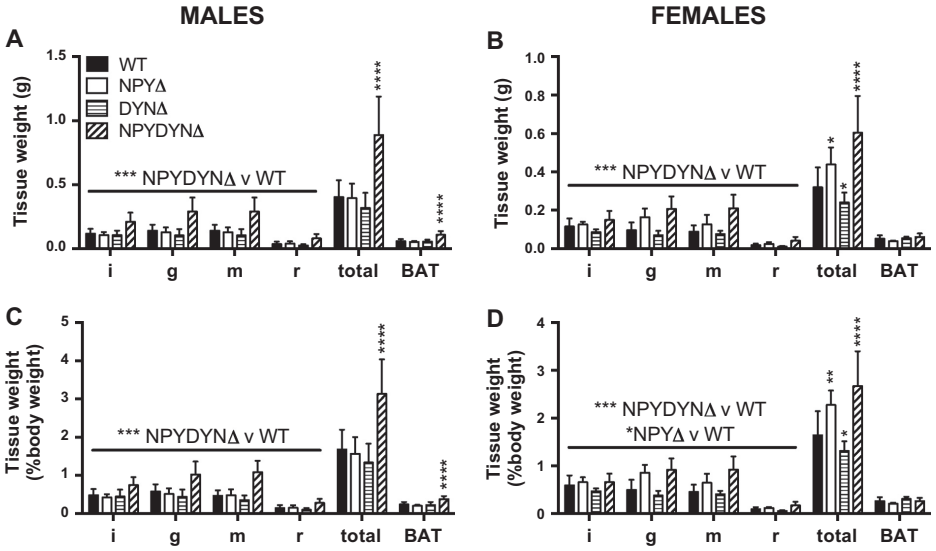

Fig. 2. Markedly increased adiposity in NPYDYNΔ mice despite no significant difference in single NPYΔ or DYNΔ mice. Weight, in grams and normalised to body weight (% body weight), of dissected white adipose tissue (WAT) and brown adipose tissue (BAT) depots in male (A, C) and female (B, D) wildtype (WT), NPYΔ, DYNΔ and NPYDYNΔ mice. Abbreviations: i, right inguinal; g, right gonadal; m, mesenteric; r, right retroperitoneal; total, summed weight of i, g, m and r WAT depots. Plotted values are means ± SEM of 6 or more mice per group. ***$P < 0.001$ and ****$P < 0.0001$ compared to WT mice.

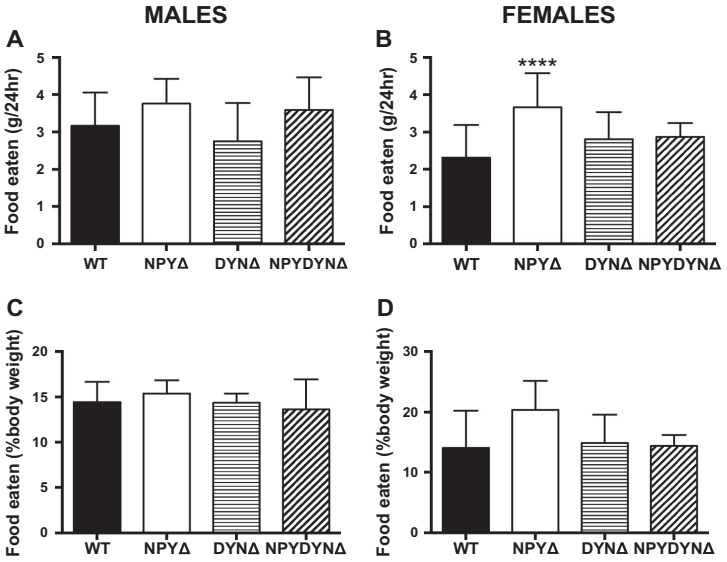

Fig. 3. Spontaneous food intake is unchanged by double NPYDYN deletion. Spontaneous 24-h food intake in male (A, C) and female (B, D) wildtype (WT), NPYΔ, DYNΔ and NPYDYNΔ mice, expressed in absolute weight (A, B) and normalised to body weight (% body weight, C, D). Plotted values are means ± SEM of 7 or more mice per group. ****$P < 0.0001$ compared to WT mice.

3.4. Deletion of NPY, dynorphin, or both neuropeptide precursors results in increased body weight loss after a 24-h fast

Directly following the 24-h fasting period, male but not female NPYΔ and DYNΔ, along with female but not male NPYDYNΔ mice lost a significantly greater amount of body weight than their wildtype counterparts (Fig. 4E–H). Weight regain during the 72-h post-fasting period was similar between NPYDYNΔ and wildtype mice of both sexes, but male NPYΔ showed a significantly delayed regain and female DYNΔ showed a significantly faster regain relative to wildtype mice (Fig. 4I, J).

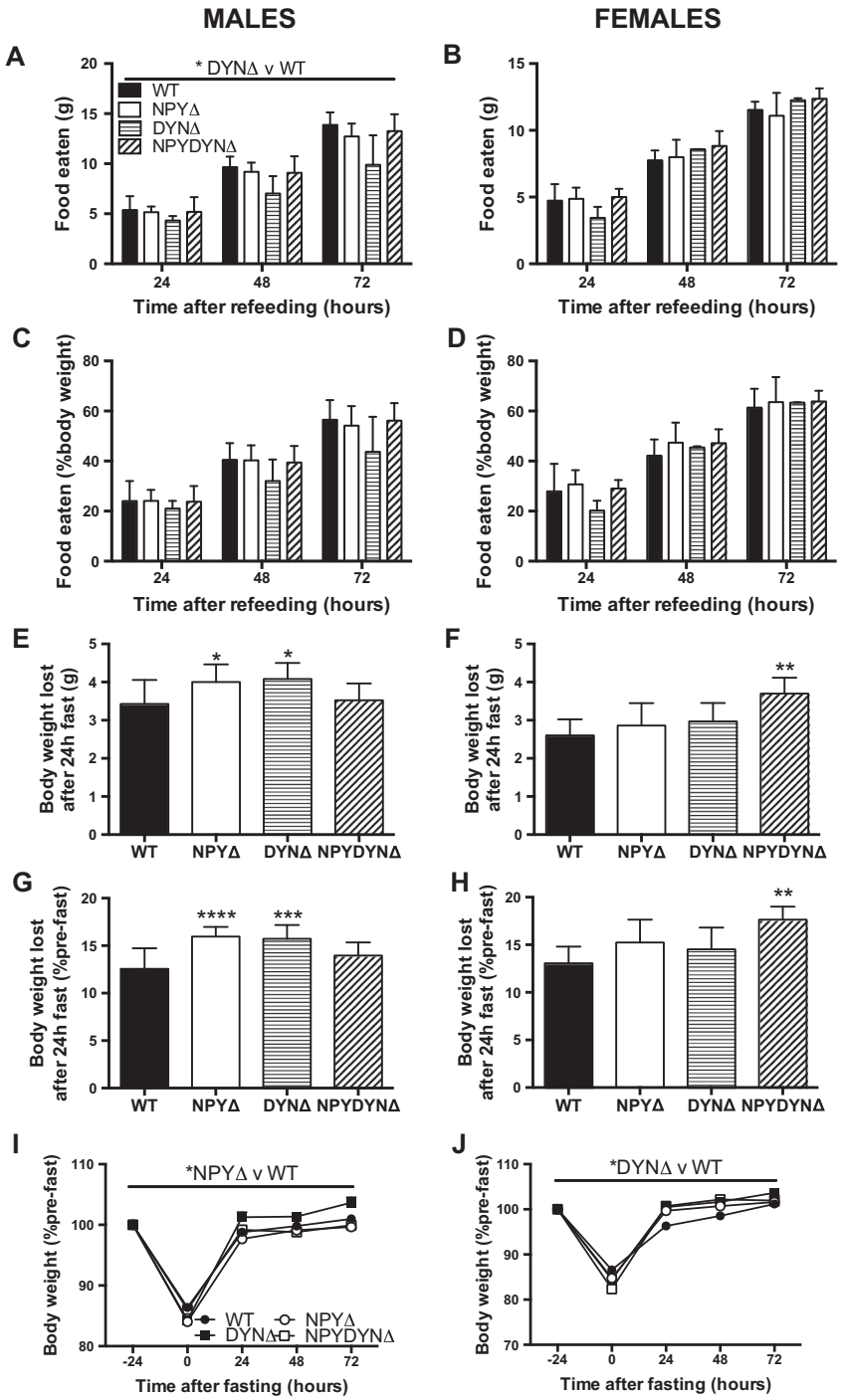

Fig. 4. No effect of double NPYDYN deletion on fasting-induced food intake. Accumulated 24-h fasting-induced food intake in absolute weight (A, B) and normalised to body weight (% body weight, C, D) of wildtype (WT), NPYΔ, DYNΔ and NPYDYNΔ mice. Body weight lost by the four groups of mice after a 24-h fast is depicted as both absolute weight (E, F) and as a percent of pre-fasting body weight (% pre-fast, G, H). Body weight was measured at the time points of fasting-induced food intake measurements in male (I) and female (J) mice. Data are presented as a percent of pre-fasting body weight (% pre-fast). Plotted values are means ± SEM of 5 or more mice per group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$ compared to WT mice.

3.5. Obese NPYDYNΔ mice do not display reductions in brown adipocyte thermogenesis markers

As noted above (Fig. 2), brown adipose tissue mass was significantly increased in male NPYDYNΔ mice. Brown adipose tissue is primarily involved in thermal regulation through its mitochondrial abundance of uncoupling protein-1 (UCP-1), the key mediator of brown adipocyte thermogenesis through its interactions with free fatty acids to release energy in the form of heat, as well as peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC1-α), which acts as a cold-inducible protein that controls adaptive thermogenesis (Fernandez-Marcos and Auwerx, 2011). To gain insight into whether NPYDYNΔ mice may have had altered thermogenesis, which could help to explain their obese phenotype, we measured protein levels of these key mediators of thermoregulation in the brown adipose tissue of our male mice. Compared to wildtype mice, NPYΔ mice displayed significantly elevated UCP-1 protein levels, whereas DYNΔ and NPYDYNΔ mice showed no significant difference (Fig. 5A). No differences were seen in PGC1-α protein content in brown adipose tissue when comparing any knockout group with that of wildtype mice (Fig. 5B).

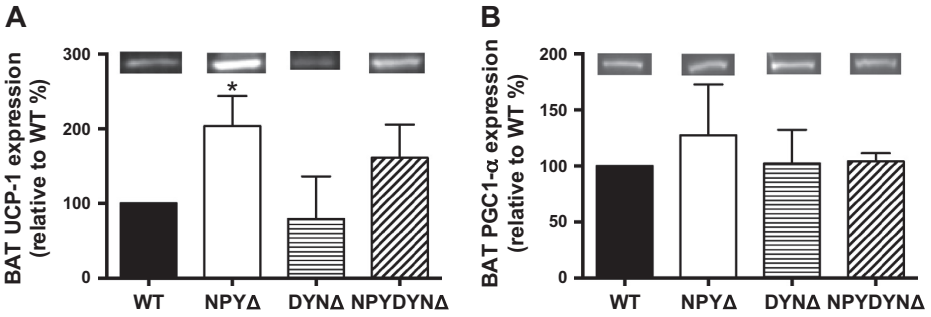

Fig. 5. Brown adipose tissue thermogenesis markers are unchanged in male NPYDYNΔ mice. Protein levels of uncoupling protein-1 (UCP-1) (A) and peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC1-α) (B) of male NPYΔ, DYNΔ and double NPYDYNΔ mice, normalised to the expression in wildtype mice, as measured by Western blot. Plotted values are means ± SEM of 3 mice per group. $^{*}P < 0.05$ compared to WT mice.

3.6. Double deletion of both NPY and dynorphin has no effect on glucose metabolism

As determined by intraperitoneal glucose tolerance tests and the resultant curves of serum glucose concentrations, and as compared to wildtype controls, double deletion of NPY and dynorphin precursors had no effect on glucose metabolism in male or female mice (Fig. 6). This was despite significant, albeit opposite, differences in these parameters in single knockout mice. Indeed, both male and female NPYΔ mice demonstrated worsened glucose tolerance relative to wildtype controls, and male but not female DYNΔ had improved glucose tolerance (Fig. 6).

4. Discussion

The results from this study demonstrate that the simultaneous ablation of both NPY and dynorphin in mice does not induce greater weight or fat losses than changes induced by single deletion of either NPY or dynorphin. In fact, double deletion of NPY and dynorphin resulted in significantly heavier mice, with corresponding and marked increases in adiposity relative to wildtype controls. This was a surprising finding as it has previously been reported, and corroborated, although not significantly in both sexes in this particular study cohort, that DYNΔ mice actually display an antiobesity effect through decreased visceral and subcutaneous adiposity levels (Sainsbury et al., 2007). Moreover, NPY knockout mice have previously been shown to exhibit either unchanged food intake (Erickson et al., 1996a and Hill and Levine, 2003), as well as unaltered body weight and/or adiposity levels (Erickson et al., 1996a and Zengin et al., 2013), or in some circumstances, including leptin deficiency (Erickson et al., 1996b), reductions in fat mass (Baldock et al., 2009 and Zengin et al., 2013). Indeed, in our current cohort of NPY knockout mice we observed no significant change from wildtype with respect to body weight or adiposity. Therefore, it can be seen that the obesity-resistant phenotype of single DYNΔ mice and the unaltered body weight and adiposity of NPYΔ mice in this study was not carried over to the double NPYDYNΔ mice, which displayed no combinatory or synergistic effect with regards to body weight and composition.

The increased body weight and adiposity seen in our NPYDYNΔ mice cannot be

explained by consistent increases in energy intake in both sexes, and do not seem to be explained by altered thermogenesis, either. NPYDYNΔ mice did not have a clearly altered food intake phenotype, when measured either under spontaneous or fasting-induced conditions, nor did they exhibit differences from wildtype mice with respect to brown adipose tissue content of the major thermogenesis markers, UCP-1 and PGC1-α. Although the weight of brown adipose tissue in male NPYDYNΔ mice was significantly increased relative to wildtype, this is likely due to an increase in the relative size of the brown adipocytes or increased white adipocyte infiltration, as opposed to functional changes in thermogenic capacity. Therefore, the markedly obese phenotype of the NPYDYNΔ mice must be due to other factors besides altered energy intake or thermogenesis, such as decreases in physical activity or energy expenditure, greater propensities to utilise carbohydrates over fats as fuel, or by the increased absorption of energy from food by the gastrointestinal tract, as has recently been reported in antibiotic-exposed mice that became obese despite no changes in food intake or energy expenditure (Cho et al., 2012). Additionally, other changes in body composition have been demonstrated in these experimental mouse models. Bone tissue makes a substantial contribution to body weight, and in both single NPYΔ and DYNΔ mouse models, indices of elevated cancellous bone mass have been reported (Baldock et al., 2012). Interestingly, NPYDYNΔ double knockout mice also exhibit a similar increase in bone volume to that of single deletion mouse models (Baldock et al., 2012). This demonstrates a relationship between dynorphin and NPY in the control of other body composition components besides adipose tissue, namely, bone.

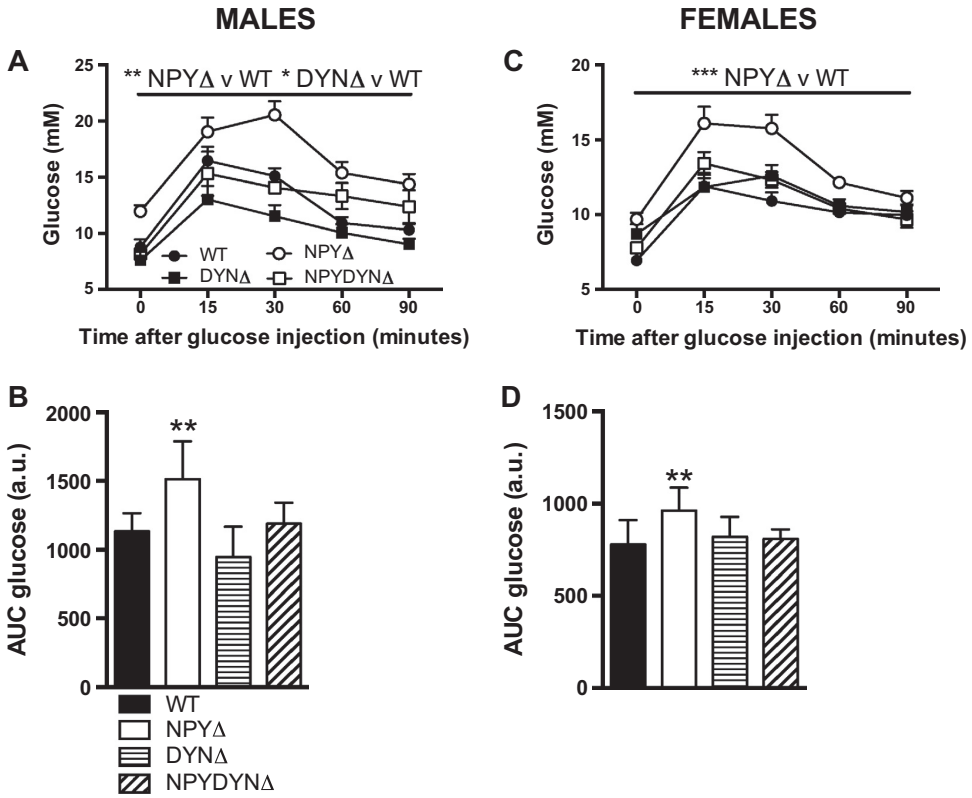

Fig. 6. Unaltered serum glucose responses to intraperitoneal glucose injection in NPYDYNΔ mice. Intraperitoneal glucose tolerance tests (1 g/kg) were conducted in 16- to 24-h fasted male (A, B) and female (C, D) wildtype (WT), NPYΔ, DYNΔ and NPYDYNΔ mice. Areas under the curve for corresponding graphs were calculated for the glucose responses (B, D). Plotted values are means ± SEM of 5 or more mice per group. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to WT mice.

The greater amount of weight lost after a 24-h fast seen in the female double NPYDYNΔ mice, which was not seen in either single NPYΔ or DYNΔ mice, could perhaps suggest a synergistic relationship between the two orexigenic pathways of NPY and dynorphin. Specifically, an additive regulatory mechanism in protecting against excess loss of body weight after food deprivation may be a specific function of these two orexigenic pathways, in which other regulatory pathways are not able to compensate for. Similarly, with regards to glucose homeostasis, additive effects of both the single NPY and dynorphin

ablation appear to occur in the NPYDYNΔ mice. Double NPYDYNΔ mice of either gender do not display an altered blood glucose time-course profile after an injection of glucose, compared to that of wildtype mice. However, both male and female NPYΔ mice demonstrated worsened glucose tolerance, and male but not female DYNΔ had improved glucose tolerance. The double NPYDYNΔ mouse model seems to respond in a manner that combines the responses of the two single NPYΔ and DYNΔ models, thus negating their opposing effects.

It is counter-intuitive that double deletion of two systems that have previously been implicated in mediating obesogenic responses – the NPY and dynorphin precursors – resulted in a markedly obese animal model. It is noteworthy that our studies specifically focused on the disruption of dynorphins. Dynorphins activate all three types of opioid receptors (μ, δ and κ), but bind with much higher preference for κ-opioid receptors (Hamilton and Bozarth, 1988), which have been implicated in the regulation of energy homeostasis (Arjune and Bodnar, 1990, Hamilton and Bozarth, 1988 and Levine et al., 1990). It is possible that other endogenous opioid system peptides, the endorphins and enkephalins, could functionally compensate for the absence of dynorphin function. Additionally, as germline knockout models exhibit compensation from other systems (Cooke et al., 1997), a double knockout mouse model, where two physiological systems have been disrupted, may experience an even stronger compensatory functional response. Indeed, it has been shown that simultaneous disruption of both Y1 and Y5 receptors, purported to be 'feeding receptors' mediating orexigenic effects of NPY, resulted in expectedly decreased food intake, yet these animals become obese (Nguyen et al., 2012). Interestingly, this phenomenon was observed even when both Y1 and Y5 receptors were deleted in an inducible, adult-onset knockout model (Nguyen et al., 2012), demonstrating a fundamental tendency for mammals to become obese. These phenomena could provide an explanation for the results seen in the double NPYDYNΔ model of this study. To overcome such developmental compensation, pharmacological antagonists against both NPY and dynorphin could be tested in obese mice.

Pharmaceuticals that target dual systems are the next step in the race to develop effective obesity pharmacotherapy. Recently, several fixed-dose combination drugs that target dual pathways to treat obesity in a multifaceted, synergistic way have either been submitted to the FDA for approval, or are in late phase clinical research and development. These drug therapies include *Qsymia*, developed by Vivus, which is a drug combining phentermine, an appetite suppressant and topiramate, an anticonvulsant; *Empatic*, marketed by Orexigen Therapeutics, a combination of bupropion, which disrupts the catecholamine system and zonisamide, also an anticonvulsant; as well as Amylin's *Symlin*, a synthetic analogue of pancreatic peptide combining amylin and metreleptin, a leptin agonist. Additionally, the aforementioned *Contrave* utilises a combination of opioid receptor antagonism and dopamine reuptake inhibition, through naltrexone and bupropion respectively. In light of our current findings, long-term dual targeting of NPY and dynorphins may not be a suitable strategy for the treatment of obesity.

In conclusion, it can be seen that combining opioid blockade with disruption of the NPY system via genetic ablation did not elicit anti-obese effects in mice, but instead resulted in marked obesity. While the targeting of multiple pathways will likely be a major part of the development of effective new obesity treatments, and while opioid antagonism has recently been increasingly investigated as a potential anti-obesity treatment, combining long-term opioid antagonism with long-term NPY-ergic blockade would seem unlikely to produce more effective anti-obesity treatments.

Author contributions

ADN: data analysis and interpretation, literature search, generation of figures, manuscript preparation; KS: data collection; CS: data interpretation, manuscript preparation; NJL; data collection; DB: data collection; LM: data interpretation, manuscript preparation; EY: data collection, manuscript preparation; RFE: data collection; LZ: data interpretation, manuscript preparation; SL: data interpretation, manuscript preparation; YCS: data analysis and interpretation, manuscript preparation; PAB: data interpretation, manuscript preparation; HH: study design, obtaining funding, data interpretation, literature search, manuscript preparation; AS: study design, obtaining funding, data analysis and interpretation, literature search, generation of figures, manuscript preparation.

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