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Central neuropeptide Y infusion and melanocortin 4 receptor antagonism inhibit thyrotropic function by divergent pathways

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Abstract

Weight loss inhibits thyrotropic function and reduces metabolic rate, thereby contributing to weight regain. Under negative energy balance there is an increase in the hypothalamic expression of both neuropeptide Y (NPY) and agouti related peptide (AgRP), the endogenous antagonist of melanocortin 4 (MC4) receptors. Both NPY and MC4 receptor antagonism reduce thyrotropic function centrally, but it is not known whether these pathways operate by similar or distinct mechanisms. We compared the time-course of effects of acute or chronic intracerebroventricular (ICV) administration of NPY (1.2 nmol acute bolus, or 3.5 nmol/day for 6 days) or the MC4 receptor antagonist HS014 (1.5 nmol bolus, or 4.8 nmol/day) on plasma concentrations of thyroid stimulating hormone (TSH) or free thyroxine (T4) in male rats pair-fed with vehicle-infused controls. These doses equipotently induced hyperphagia in acute studies, reduced latency to feed, and increased white adipose tissue mass after 6 days of infusion. Acute central NPY but not HS014 administration significantly reduced plasma TSH concentrations within 30–60 min and plasma free T4 levels within 90–120 min. These inhibitory effects were sustained for up to 5–6 days of continuous NPY infusion. HS014 induced a transient decrease in plasma free T4 levels that was observed only after 1–2 days of continuous ICV infusion. While both NPY and HS014 significantly increased corticosteronemia within an hour after ICV injection, the effect of NPY was significantly more pronounced and was sustained for up to 4 days of administration. Both NPY and HS014 significantly decreased the brown adipose tissue protein levels of uncoupling protein-3. We conclude that central NPY and MC4 antagonism decrease thyrotropic function via partially distinct mechanisms with different time courses, possibly involving glucocorticoid effects of NPY. MC4 receptor antagonism increases adiposity via pathways independent of increased food intake or changes in circulating concentrations of TSH, free T4 or corticosterone.

1. Introduction

A major challenge for the treatment of obesity is that loss of just 5–10% of body weight leads to metabolic changes that inhibit further weight loss. Overweight or obese people who lost weight by moderate or severe energy restriction (with or without physical activity) showed significant decreases in circulating concentrations of the most active thyroid hormone free triiodothyronine (T3) and/or a significant increase in that of the inactive hormone, reverse T3, as well as significant reductions in circulating concentrations of thyroid stimulating hormone (TSH) and free thyroxine (T4), the precursor to T3 (Hukshorn et al., 2003a, Hukshorn et al., 2003b, Rosenbaum et al., 2000, Weinsier et al., 2000, Wadden et al., 1990, Naslund et al., 2000 and Douyon and Schteingart, 2002). As thyroid hormones are major regulators of energy expenditure, acting on peripheral tissues to directly influence cellular metabolism (Silva, 2003) as well as on hypothalamic sites to regulate AMP-activated protein kinase, fatty acid metabolism and subsequent sympathetic nervous output (Lopez et al., 2010), reduced thyroid function likely contributes to the concomitant reduction in metabolic rate or energy expenditure (Weinsier et al., 2000, Rosenbaum et al., 1997, Leibel et al., 1995, Westerterp-Plantenga et al., 2001, Westerterp-Plantenga et al., 2004, Hukshorn et al., 2003a, Hukshorn et al., 2003b, Menozzi et al., 2000, Martin et al., 2007 and Sainsbury and Zhang, 2010). The weight loss-induced drop in energy expenditure is a significant predictor of weight regain (Pasman et al., 1999 and Goran, 2000), so insights into mechanisms for decreased thyroid function and energy expenditure could lead to improved weight loss interventions.

The decreases in thyroid function and metabolic rate that occur during negative energy balance are likely to be mediated, at least in part, by the action of neuropeptide Y (NPY) and melanocortin receptors in the hypothalamus (Lechan and Fekete, 2006). NPY is an orexigenic peptide (Stanley et al., 1985 and Clark et al., 1984) synthesized by neurons within the arcuate nucleus of the hypothalamus (Bai et al., 1985). These NPY-ergic neurons also synthesize another orexigenic agent, agouti related peptide (AgRP) (Ollmann et al., 1997), which antagonizes melanocortin 3 (MC3) and melanocortin 4 (MC4) receptors expressed in the brain (Ollmann et al., 1997). The NPY–AgRP synthesizing neurons in the arcuate nucleus are distinct from the cells that express proopiomelanocortin (Elias et al., 1998), the pre-cursor for the anorexic alpha-melanocyte stimulating hormone (α-MSH), which acts as an agonist on brain MC3 and MC4 receptors (Mountjoy et al., 1992 and Adan et al., 1994). NPY–AgRP and POMC-expressing neurons in the arcuate nucleus project to the paraventricular nucleus (PVN) (Baker and Herkenham, 1995 and O'Donohue et al., 1979), where they exert stimulatory (NPY and AgRP) (Stanley et al., 1985 and Clark et al., 1984) or inhibitory (α-MSH) (Thiele et al., 1998 and Brown et al., 1998) effects on food intake. During energy restriction in rodents, the protein or mRNA levels of NPY and AgRP are increased in the hypothalamus whereas the mRNA expression of proopiomelanocortin is reduced (Sainsbury and Zhang, 2010). This change likely has important effects on the hypothalamo-pituitary thyroid axis, as NPY, AgRP, and α-MSH-containing neuronal endings have been detected in close association with neurons in the PVN that synthesize thyrotropin-releasing hormone (TRH) in rodents (Legradi and Lechan, 1999) and in humans (Mihaly et al., 2000). Moreover, TRH-expressing neurons in the PVN of the mouse are known to express Y1 receptor-like immunoreactivity (Broberger et al., 1999). Unlike the MC3 receptor, MC4 receptor mRNA (Mountjoy et al., 1994) as well as Y1 and Y5 receptor immunoreactivity (Wolak et al., 2003) are found in both parvicellular and magnocellular neurons of the PVN as recently reviewed (Lechan and Fekete, 2006), suggesting direct effects of NPY and melanocortins on the thyrotropic axis at the level of the hypothalamus. Indeed, central administration of NPY, Y1 or Y5 receptor agonists, AgRP or MC4 receptor antagonists to normal rodents significantly reduces function of the thyrotropic axis, indicated by reductions in expression of TRH in the PVN, and a decrease in circulating concentrations of TSH and T3 or T4 (Fekete et al., 2001, Fekete et al., 2002a, Fekete et al., 2002b, Small et al., 2001 and Kim et al., 2000). MC4 receptors are critical for the inhibitory effects of AgRP on thyroid function, as the effects were not seen in MC4 receptor knockout mice (Fekete et al., 2004). Conversely, central injection of MC3 or MC4 receptor agonists such as α-MSH or a stable analog of α-MSH increased circulating TSH (Kim et al., 2000) or free T4 concentrations (Fekete et al., 2000) in fasting rodents, probably via direct actions which stimulate TRH expression and release in the PVN (Kim et al., 2000 and Fekete et al., 2000). Further evidence for a physiological role of MC4 in the regulation of thyroid function is the recent *in vivo* discovery that thyroid hormones inhibit MC4 receptor expression in brain nuclei known to regulate thyroid function or energy homeostasis, namely the PVN, arcuate nucleus of the hypothalamus and brain stem, via their receptor interactions with thyroid hormone-response elements on the MC4 receptor gene (Decherf et al., 2010). This negative feedback loop, whereby MC4 receptor activation stimulates thyroid hormone release which in turn down-regulates MC4 receptor expression, would serve to limit the catabolic effects of MC4 receptor agonism (Decherf et al., 2010). Taken together, these collective findings suggest that alterations in endogenous NPY and melanocortin tonus could contribute to the decreased thyroid function (and subsequently reduced energy expenditure) that have been consistently observed during conditions of negative energy balance, such as during weight loss in obese individuals.

NPY, AgRP and their receptors share not only overlapping distribution patterns, but also overlapping functions. While such functional redundancy could contribute to species survival during prolonged period of food restriction, it poses a particular challenge for voluntary weight loss strategies. Indeed, recent clinical trials in humans of MK-0493, an MC4 receptor agonist, revealed a slight but significant decrease in food intake and a modest decrease in body weight from baseline, with no significant effect on body weight after 12–18 weeks (Krishna et al., 2009). In light of the redundancies in systems that regulate energy homeostasis or thyroid function, we aimed to investigate the mechanisms by which NPY and AgRP may inhibit activity of the hypothalamo-pituitary–thyrotropic axis, and to determine whether this inhibition is occurring through distinct or overlapping mechanisms. To this end we investigated the time course of effects of acute versus chronic intracerebroventricular

(ICV) infusion of NPY or the MC4 receptor antagonist, HS014, on thyrotropic function in rats.

2. Materials and methods

2.1. Experimental animals

All procedures were approved by the Animal Experimentation Ethics Committee of the Garvan Institute/St. Vincent's Hospital and are in keeping with the National Health and Medical Research Council of Australia's guidelines on animal experimentation. Male Wistar rats were purchased from Animal Resources Centre (Perth, Australia) at weights of 250–280 g. They were housed in groups in plastic cages on pellet paper bedding and under conditions of controlled temperature (23 °C) and illumination (6:00–18:00 h). They were allowed *ad libitum* access to standard laboratory chow (Norco Stockfeeds, South Lismore, Australia) and water, unless otherwise stated.

2.2. Placement of chronic intracerebroventricular (ICV) and jugular cannulae

Animals were anesthetized with an intraperitoneal injection of ketamine/xylazine at 60 and 10 mg/kg, respectively (Mavlab, Brisbane, Australia, and Troy Laboratories, Sydney, Australia) for the placement of a cannula in the right lateral cerebral ventricle (Sainsbury et al., 1997), which was used for all ICV administrations. Another cannula was placed in the right jugular vein for blood sampling. Rats received an intramuscular injection of penicillin at 0.3 mg/kg (Troy Laboratories, Sydney, Australia) as well as subcutaneous analgesic, buprenorphine (Temgesic, 0.025 mg/kg, Reckitt and Coleman, Hull, UK) and were left to recover to pre-surgery weights (7–10 days) in individual cages, with daily handling to minimize stress.

2.3. Acute ICV injection of NPY and HS014

A subset of rats were used in acute (2–4 h) studies following bolus ICV injection of porcine NPY (1.2 nmol, Auspep, Melbourne, Australia), or the synthetic MC4 receptor antagonist HS014 (1.5 nmol, Auspep, Melbourne, Australia) or vehicle (5 mL 0.9% NaCl) for control rats. Injections were made over 60 s, and animals were then returned to their cages. Blood samples (0.3 mL) were collected from the jugular cannula at 0, 7.5, 15, 30, 60, 90, and 120 min after ICV injections using sodium citrate (6.1 mg/mL in 0.9% NaCl) as an anticoagulant. Food was not available during the sampling period. Plasma was frozen at −20 °C until subsequent analysis as described below.

2.4. Chronic ICV infusion of NPY and HS014

Rats were anesthetized with halothane (Veterinary Companies of Australia, Artarmon, Sydney, Australia) for implantation of subcutaneous osmotic minipumps (model 2001, Alza Corporation, Palo Alto, CA, US) (Sainsbury et al., 1997) which were connected via polyethylene tubing to the ICV cannula for central infusion of porcine NPY (3.5 nmol/day), HS014 (4.8 nmol/day), or vehicle (24 μL/day of 0.9% NaCl) for control rats. Food intake of the NPY- and HS014-infused rats was restricted to that of vehicle-infused animals (30–32 g/day) to ensure that any differences observed were not due to increased food intake. Rats were fasted for 2–3 h (from ~9.30–12.30 h) prior to collection of blood samples (0.3 mL) from the jugular cannula between 11:30 and 12:30 h each day using sodium citrate (6.1 mg/mL in 0.9% NaCl) as an anticoagulant. All plasma samples were frozen and stored at −20 °C until analysis.

2.5. Plasma analyses

Plasma concentrations of glucose were measured using a commercial kit (glucose oxidase method, Trace Scientific, Melbourne, Australia). Commercial radioimmunoassay kits were used for the determination of plasma insulin, leptin (Linco Research, St. Louis, MO, US), corticosterone, free T4, and TSH levels (ICN Biomedicals, Costa Mesa, CA, US), except for chronic infusion studies, where plasma TSH concentrations were estimated by chemiluminescent immunometric assay (CLIMA) on Immulite® 2000 (DPC Biermann GmbH, Friedberg, Germany) and free T4 was estimated by Microparticles Enzyme Immunoassay on AxSYM® (Abbott Park Illinois, USA). For measurement of TSH and Free T4 levels, plasma was combined from two time points from each rat (i.e. t = 30 and t = 60 min sample; t = 90 and t = 120 min sample; t = 1 and t = 2 day sample; t = 5 and t = 6 day sample), in order to have sufficient plasma volume for the assay.

2.6. Tissue analyses
After 6 days of ICV infusion, rats were anaesthetized with pentobarbitone sodium (Nembutal; Abbott laboratories, Sydney, Australia), the interscapular brown adipose tissue (BAT) was removed, weighed, rapidly frozen on liquid nitrogen and stored at −80 °C until subsequent analysis of UCP3 protein levels as previously described (Molero et al., 2004). The thyroid gland was also removed and weighed. The right inguinal and right epididymal white adipose tissue depots were removed and weighed, as was the mesenteric white adipose tissue depot.

2.7. Statistics
Statistics were performed using Statview version 4.5 (Abacus Concepts, CA, US). Repeated measures or factorial ANOVA tests were used for the statistical analysis of results, with Fisher's post hoc tests where appropriate. Data are expressed as mean ± SEM (or mean ± SD where the graph shows groups with less than six animals), and the results were considered statistically significant if $P < 0.05$.

3. Results

3.1. Acute central NPY but not HS014 injection decreases circulating thyroid hormone concentrations and increases insulinemia
In order to investigate immediate effects of central NPY or MC4R antagonist administration on thyroid function, we measured changes in circulating thyroid hormone concentrations in response to acute ICV injection of NPY or HS014 in rats that did not have access to food. There was no significant difference among the three groups of rats with respect to body weight at the time of injection (data not shown). We have shown previously that the acute doses of NPY (1.2 nmol) and HS014 (1.5 nmol) chosen for this study were equipotent in that both agents produced an equivalent, 10-fold increase in total food intake in the 4 h after ICV injection compared to vehicle-injected controls (Baran et al., 2002). Despite comparable actions on food intake in rats that were allowed to eat, only NPY injection had effects on thyroid function. Acute ICV administration of NPY resulted in a significant reduction in plasma concentrations of TSH relative to vehicle-injected controls at 30–60 min post-injection, and this effect was sustained at 90–120 min post-injection (Fig. 1A). Acute ICV NPY injection also led to a significant reduction in plasma free T4 concentrations compared to vehicle-injected control rats at 90–120 min after injection (Fig. 1B). In contrast to these inhibitory effects of NPY, there were no effects of acute ICV HS014 injection on the plasma concentrations of TSH or Free T4 (Fig. 1).

Acute ICV NPY administration is known to modulate circulating concentrations of corticosterone and insulin (Sainsbury et al., 1996 and Wisialowski et al., 2000), both of which – in addition to thyroid hormones – influence energy homoestasis. Moreover, corticosterone and insulin status can have significant effects on thyroid hormone concentrations (Kakucska et al., 1995, Alkemade et al., 2005, Nicoloff et al., 1970, Gonzalez et al., 1980 and Mitsuma and Nogimori, 1982). Therefore, in order to determine whether changes in insulin or corticosterone levels may have contributed to these changes in thyroid hormone concentrations, we investigated circulating insulin and corticosterone concentrations in NPY- and HS014-injected rats. Both peptides induced a significant increase in plasma corticosterone levels relative to vehicle-injected control rats within the first 120 min of injection, and this effect was significantly more pronounced in NPY- than in HS014-injected rats (Fig. 2A). While acute ICV injection of neither NPY nor HS014 had any significant effect on glycemia (Fig. 2B), acute central NPY but not HS014 administration significantly increased insulinemia within the 2 h after injection (Fig. 2C).

3.2. Chronic ICV NPY or HS014 administration both stimulate appetite and induce obesity
As acute ICV HS014 administration did not influence circulating thyroid hormone concentrations, we sought to determine whether longer-term central administration (up to 6 days) would have effects on the thyrotropic axis. In these chronic studies, all animals were pair fed the same amount as vehicle-infused controls in order to eliminate effects of NPY- or HS014-induced hyperphagia on endocrine parameters. Therefore, latency to feed was measured as an index of biological potency of the selected doses of NPY and HS014. In all of the NPY- and HS014-infused rats, latency to feed was less than 30 s on each of days 2–6 of infusion compared with greater than 60 s in vehicle-infused control rats, indicating that both

peptides stimulated appetite in all experimental rats.

There was no significant difference between vehicle-, NPY-, or HS014-infused rats with respect to body weight before or after the 6 days of ICV infusion (Table 1). This is consistent with data showing that whereas chronic ICV NPY or MC4 receptor antagonist infusion significantly increased body weight in *ad libitum*-fed rats, no such increase is observed when hyperphagia is prevented by pair-feeding with vehicle-infused controls (Baran et al., 2002).

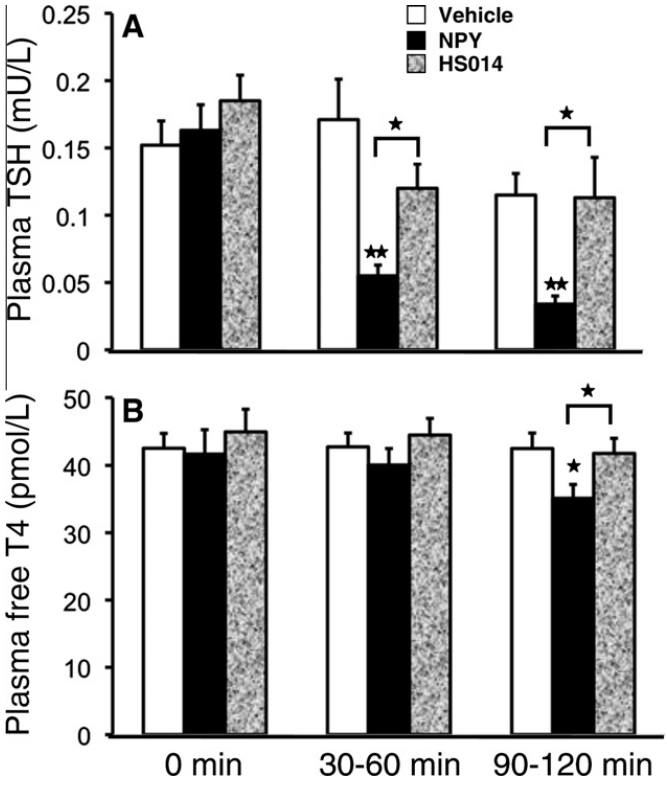

Fig. 1. Acute central administration of NPY but not the melanocortin 4 receptor antagonist HS014 reduces circulating thyroid hormone concentrations. Plasma concentrations of (A) thyroid stimulating hormone (TSH) and (B) free thyroxine (free T4) at various times after acute intracerebroventricular injection of NPY (1.2 nmol) or HS014 (1.5 nmol) compared with vehicle injected control rats (5.0 μL of 0.9% NaCl). Plotted values are means ± SEM of 8–10 rats per group. $^{*}P < 0.05$ or $^{**}P < 0.01$ versus vehicle-injected control rats at the same time point, or the comparison shown by horizontal bars.

Table 1
Effect of chronic central administration of NPY or the melanocrotin 4 receptor antagonist HS014 on body weight, white adipose tissue mass, thyroid gland weight and brown adipose tissue mass.

	Vehicle	NPY	HS014
Body weight prior to start of intracerebroventricular infusion (g)	326 ± 3.7	338 ± 11.3	325 ± 3.9
Body weight (g)	323 ± 9	335 ± 10	328 ± 3
Inguinal white adipose tissue weight (% of body weight)	0.41 ± 0.03	0.45 ± 0.06	0.54 ± 0.06*
Epididymal white adipose tissue weight (% of body weight)	0.28 ± 0.02	0.34 ± 0.03	0.41 ± 0.05*
Plasma glucose (mM)	6.4 ± 0.5	6.1 ± 0.3	6.1 ± 0.2
Weight of thyroid gland (% of body weight)	0.19 ± 0.02	0.18 ± 0.01	0.18 ± 0.01
Interscapular brown adipose tissue mass (% of body weight)	0.06 ± 0.01	0.11 ± 0.02*	0.09 ± 0.01*

Unless otherwise stated, measurements were made after 6 days of intracerebroventricular infusion of NPY (3.5 nmol/day), HS014 (4.8 nmol/day) or vehicle for control rats (24 μL/day of 0.9% NaCl). Data are means ± SEM of 6–8 rats per group.
* $P < 0.05$ versus vehicle-infused control rats.

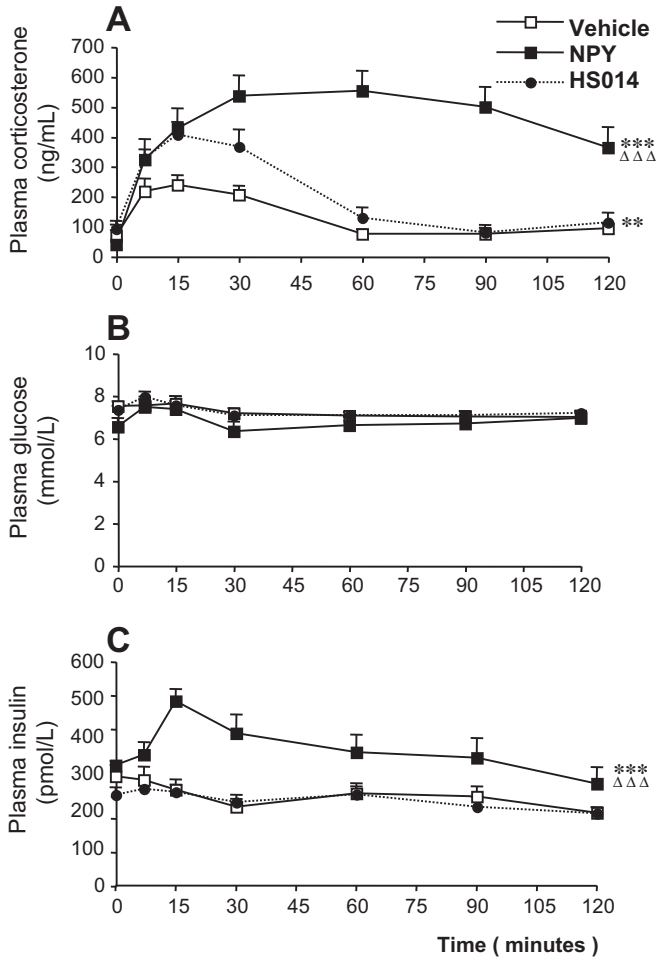

Fig. 2. Acute central administration of NPY or the melanocortin 4 receptor antagonist HS014 stimulates corticosteronemia in rats, but only NPY increases insulinemia. Plasma concentrations of (A) corticosterone, (B) glucose and (C) insulin in the 120 min after acute intracerebroventricular injection of NPY (1.2 nmol) or HS014 (1.5 nmol) compared with vehicle-injected control rats (5.0 μL of 0.9% NaCl). Plotted values are means ± SEM of 8–10 rats per group. $^{**}P < 0.01$ and $^{***}P < 0.001$ versus the curve for vehicle-injected controls; $\Delta\Delta\Delta$ $P < 0.001$ versus the curve for HS014-injected rats.

Investigation of white adipose tissue depot weights after 6 days of ICV infusion showed that NPY and HS014 infusion induced similar increases in weight of the mesenteric white adipose tissue (WAT) depot (Fig. 3A), as well as similar increases in the combined weight of all WAT depots investigated (right inguinal, right epididymal and mesenteric, Fig. 3B). Additionally, after 6 days of ICV infusion, both NPY- and HS014-infused rats exhibited significant increases in plasma concentrations of leptin (Fig. 3C), in keeping with the development of obesity in both groups of animals. Despite similarity of the overall increase in adiposity, there was a difference in fat distribution between NPY- and HS014-infused animals; only the latter group showed significant increases in relative weight of lower body fat depots, with inguinal and epididymal WAT depots being significantly heavier in HS014- but not in NPY-infused rats relative to vehicle-infused animals (Table 1). This finding suggests that while central NPY infusion particularly enhances mesenteric fat accumulation, MC4 receptor antagonism with HS014 promotes fat accumulation in both central and gluteal fat depots.

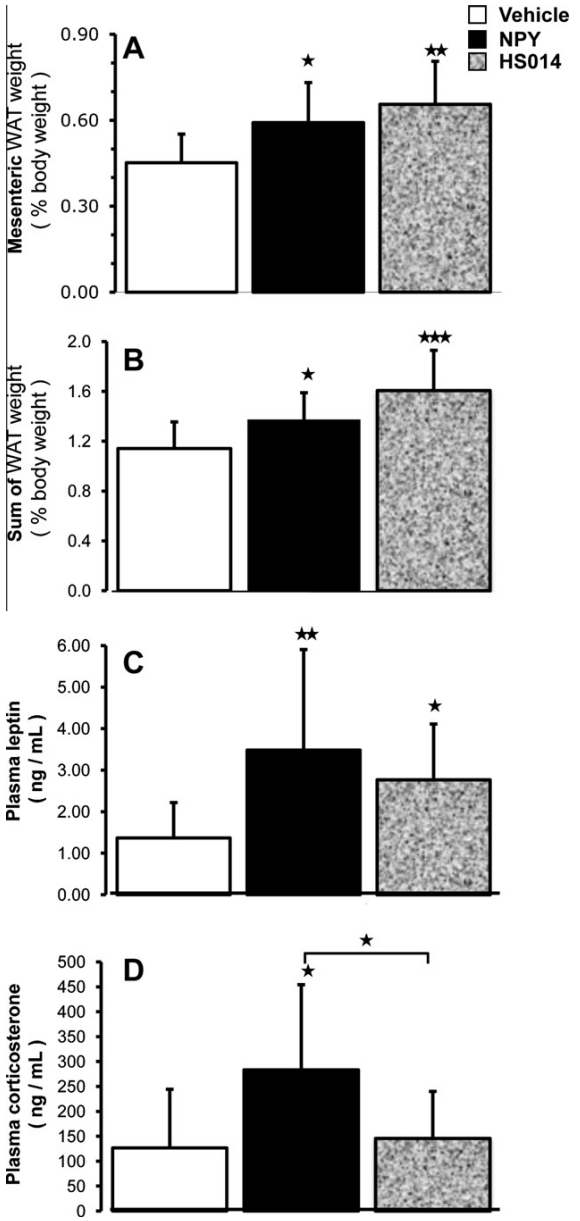

Fig. 3. Chronic central administration of NPY or the melanocortin 4 receptor antagonist HS014 increase adiposity and leptinemia in rats, but only NPY increases corticosteronemia. (A) Mass (as a percent of body weight) of mesenteric white adipose tissue (WAT) and (B) the summed mass (as a percent of body weight) of mesenteric, right epididymal and right inguinal WAT depots after 6 days of intracerebroventricular infusion of NPY (3.5 nmol/day) or HS014 (4.8 nmol/day) compared with vehicle-infused control rats (24 μL/day of 0.9% NaCl). Plasma concentrations of (C) leptin and (D) corticosterone after 4 days of intracerebroventricular infusion of NPY or HS014 compared with vehicle-infused controls as described above. Plotted values are means ± SD of 4–10 rats per group. $^{*}P < 0.05$, $^{**}P < 0.01$ or $^{***}P < 0.001$ versus vehicle-infused control rats or the comparison shown by horizontal bars.

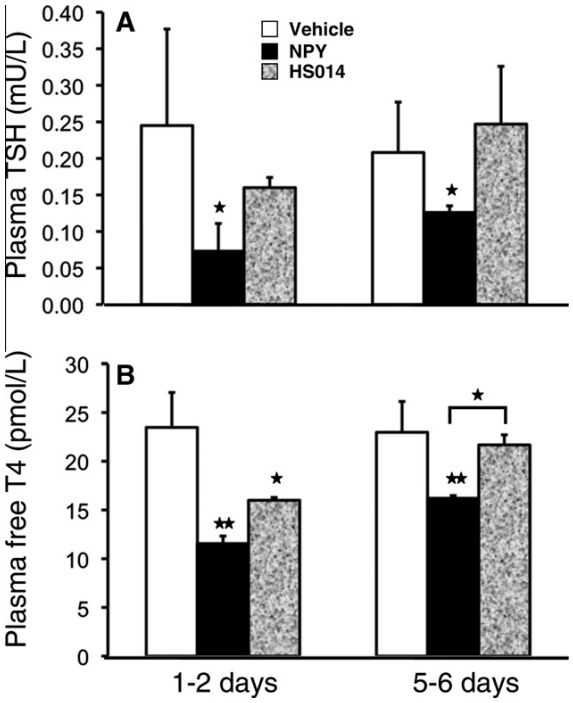

Fig. 4. Chronic central administration of NPY but not the melanocrotin 4 receptor antagonist HS014 results in a sustained reduction in circulating thyroid hormone concentrations. Plasma concentrations of (A) thyroid stimulating hormone (TSH) and (B) free thyroxine (free T4) after 1–2 or 5–6 days of intracerebroventricular infusion of NPY (3.5 nmol/day) or HS014 (4.8 nmol/day) compared with vehicle-infused control rats (24 μL/day of 0.9% NaCl). Plotted values are means ± SD of 4–10 rats per group. $^{*}P < 0.05$ or $^{**}P < 0.01$ versus vehicle-infused control rats at the same time point, or the comparison shown by horizontal bars.

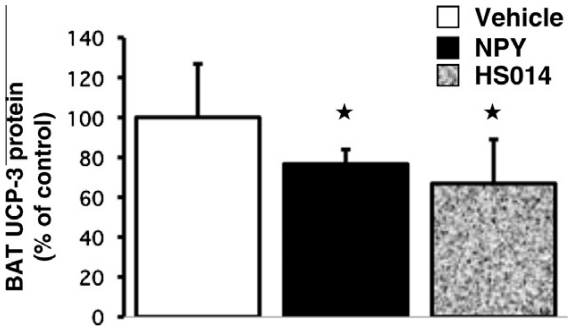

Fig. 5. Chronic central administration of NPY or the melanocortin 4 receptor antagonist HS014 similarly reduces uncoupling protein 3 (UCP-3) protein levels in brown adipose tissue. Protein levels of UCP-3 in brown adipose tissue of rats after 6 days of intracerebroventricular infusion of NPY (3.5 nmol/day) or HS014 (4.8 nmol/day), expressed as a percentage of values in vehicle-infused control animals (24 μL/day of 0.9% NaCl). Plotted values are means ± SD of 4–6 rats per group. $^{*}P < 0.05$ versus vehicle-infused control rats.

3.3. Chronic ICV NPY but not HS014 infusion results in a significant increase in corticosteronemia and a sustained reduction in circulating thyroid hormone concentrations

Despite the similar potency of these doses of NPY and HS014 on appetite and overall fat accumulation, chronic ICV administration of NPY and HS014 induced differential effects on circulating corticosterone concentrations and thyrotropic function. Whereas NPY induced a significant increase in corticosteronemia relative to vehicle treatment after 4 days of ICV infusion, no such increase in corticosteronemia was seen in HS014-infused rats (Fig. 3D). Neither NPY nor HS014 infusion had any significant effect on glycemia (data not shown). NPY induced significant decreases in plasma concentrations of TSH and free T4 that were observed at 1–2 days of ICV infusion and sustained until at least 5–6 days of infusion (Fig. 4).

In contrast, HS014 did not induce any significant effects on plasma TSH concentrations, albeit there was a 35% reduction from vehicle-infused control values at 1–2 days after infusion (Fig. 4A). After 1–2 days of ICV HS014 infusion there was a significant reduction in plasma free T4 concentrations, but this effect was normalized in comparison to vehicle-infused control values by 5–6 days of ICV HS014 infusion (Fig. 4B). This lack of effect of HS014 on plasma TSH or free T4 concentrations at 5–6 days of ICV infusion occurred despite the fact that HS014 promoted fat accumulation at least as efficiently as NPY and continued to stimulate appetite for at least 6 days of ICV infusion. There were no differences among groups with respect to the weight of the thyroid gland at the end of 6 days of ICV infusion (Table 1).

These data suggest that thyroid function in rats is inhibited by chronic central administration of NPY but not by that of the melanocortin 4 receptor antagonist HS014. It is important to note, however, that overall thyroid function in individual tissues can alter even when the circulating concentrations of thyroid hormones are unchanged, due to local differences in conversion of free T4 to the more active hormone, T3, or to the less active hormone, reverse T3, by tissue deiodinase enzymes as reviewed (Bianco et al., 2002). In order to gain insight into effects of chronic central NPY versus HS014 infusion on thyroid function in brown adipose tissue, an important site of energy metabolism, the protein levels of uncoupling protein 3 (UCP-3) in this tissue was determined, since the expression and activity of UCP-3 is known to be regulated by thyroid function (Lanni et al., 2003 and Flandin et al., 2009). The weight of brown adipose tissue depots were significantly increased in both NPY- and HS014-infused rats relative to controls (Table 1). As shown in Fig. 5, brown adipose tissue UCP-3 protein levels were significantly decreased in both NPY- and HS014-infused rats relative to vehicle-infused controls after 6 days of ICV administration. Previous work from our group and others has shown that 3–7 days central NPY, HS014 or AgRP infusion in rats also results in significant decreases in the mRNA expression of brown adipose tissue UCP-1 (Baran et al., 2002, Fekete et al., 2002a and Fekete et al., 2002b), another protein that is regulated by thyroid function. Taken together, these data do not suggest that NPY and HS014 have differential effects on overall thyroid function within brown adipose tissue.

4. Discussion

This data shows that NPY and MC4 receptor antagonism in the central nervous system inhibit activity of the thyrotropic axis via at least partially divergent mechanisms with markedly different time-courses of action. Whereas the effect of ICV NPY to reduce plasma TSH and free T4 levels is rapid in onset (within 30 min) and sustained for at least 5–6 days of continuous ICV infusion, the effect of central MC4 receptor antagonism is slower in onset and more transient, being apparent only after 1–2 days of ICV HS014 infusion. Additionally, whereas acute or chronic ICV NPY administration induced pronounced hypercorticosteronemia and hyperinsulinemia, HS014 induced a modest rise in corticosteronemia only during the first hour after acute ICV injection. As glucocorticoids inhibit activity of the thyrotropic axis (Kakucska et al., 1995, Alkemade et al., 2005 and Nicoloff et al., 1970), this finding may contribute to the observed differences in effects of NPY versus HS014 on plasma TSH and free T4 concentrations. Despite these patent dissimilarities in hormonal responses to central administration of NPY or the MC4 receptor antagonist HS014, both peptides resulted in marked and significant increases in adiposity and leptinemia after chronic infusion, even though pair feeding with vehicle-infused control animals prevented NPY- and HS014-induced hyperphagia. Additionally, both peptides inhibited protein levels of UCP-3 in brown adipose tissue after chronic central infusion, suggesting equipotent down-regulation of thyroid function, sympathetic activity, or both, in this tissue. Taken together, these findings suggest that; NPY and MC4 receptor antagonism inhibit thyroid function via different mechanisms; elevations in hypothalamic NPY expression play a stronger role in repressing circulating thyroid hormone concentrations compared to central MC4 receptor antagonism; MC4 receptor antagonism can increase adiposity via mechanisms that are independent of hyperphagia and long-term inhibition of the thyrotropic axis.

Previous studies have shown that 3 days of ICV injection or continuous infusion of the MC3/MC4 receptor antagonist AgRP into *ad libitum*-fed Sprague Dawley rats or mice suppresses circulating levels of T3 and T4 as well as hypothalamic pro-TRH mRNA in the PVN with no significant changes in circulating TSH levels (Fekete et al., 2002a, Fekete et al., 2002b and Fekete et al., 2004), consistent with our data of a significant reduction in plasma

free T4 but not TSH concentrations after 1–2 days of continuous ICV infusion of the MC4 receptor antagonist HS014. The current findings in pair fed rats extend these studies to show that the effect of MC4 receptor antagonism to reduce thyroid hormone levels is not dependent upon hyperphagia. Moreover, we have shown that the effect of MC4 receptor antagonism on thyroid hormone levels does not persist with longer infusion times (5–6 days). This is in keeping with the observation that people with early onset obesity and mutations in the MC4 receptor gene exhibit circulating free T4 levels within the normal range, with less than ten percent of these individuals showing only slight perturbations in circulating TSH levels (Farooqi et al., 2003). However, one study showed that seven daily ICV injections of AgRP into *ad libitum*- or pair fed Wistar rats significantly decreased plasma TSH concentrations. This discrepancy could be due to a possible contribution of MC3 receptors to longer-term effects of AgRP on the thyrotropic axis, since MC3 and MC4 receptors have differential effects on TRH release from hypothalamic explants *in vitro* (Kim et al., 2002). In contrast to unsustained effects of specific MC4 receptor antagonism as shown here, it is clear from this study that NPY has a sustained inhibitory effect on the thyrotropic axis.

The current findings extend previous knowledge of NPY and melanocortin regulation of thyroid function by demonstrating that while NPY may be involved in long-term repression of thyroid function under conditions such as chronic energy restriction, the shorter-lived MC4 receptor-mediated effect may serve to inhibit thyroid function only acutely in response to such stimuli. Indeed, new evidence suggests transience not only of the inhibitory effect of MC4 receptor antagonism on thyroid function, as shown in the present study, but also of the stimulatory effect of MC4 receptor agonism on this axis (e.g. by endogenous α-MSH). In keeping with this, thyroid hormones inhibit MC4 receptor expression *in vivo* in the hypothalamus and brain stem via thyroid hormone receptor interactions with thyroid hormone-response elements in the MC4 receptor gene (Decherf et al., 2010). The physiological consequence of this could be that α-MSH/MC4 receptor-mediated increases in thyroid hormone action (as would be expected under conditions of energy excess) would in turn down-regulate central MC4 receptors, thereby not only limiting the duration of thyroid stimulation by α-MSH but also acting to promote positive energy balance via other effects of MC4 receptor down-regulation, notably hyperphagia (Decherf et al., 2010). This finding reflects the complexity and redundancy of mechanisms limiting catabolic and promoting anabolic states.

NPY and MC4 receptor antagonism have been hypothesized to influence activity of the thyrotropic axis by reducing cellular cAMP concentrations leading to reduced phosphorylation of CREB and reduced transcription of the TRH gene as recently reviewed (Lechan and Fekete, 2006). However, the rapidity of the effect of ICV NPY infusion on plasma TSH levels (within 30–60 min after acute ICV injection) suggests that NPY induces at least some of its effects on plasma TSH levels via mechanisms independent of increased transcription of the TSH gene. Additionally, while the current data do not preclude the possibility that NPY and MC4 receptor antagonism may reduce plasma free T4 levels via reductions in intracellular cAMP levels and subsequent inhibition of TSH gene transcription, other mechanisms must also be at play to account for the different time courses of effects.

It is possible that the pronounced and persistent hypercorticosteronemia observed in NPY- but not HS014-infused rats contributed to the differential effects on thyroid hormone concentrations. Adrenalectomy in rats increases pro-TRH mRNA levels in the PVN, whereas administration of exogenous corticosterone or dexamethasone to rats has the opposite effect (Kakucska et al., 1995). Similar effects appear to be at play in humans, since people that had been treated with glucocorticoids until the time of death showed a significant decrease in TRH mRNA levels in the PVN compared to control subjects (Alkemade et al., 2005), and glucocorticoid excess suppresses the secretion of TSH in humans (Nicoloff et al., 1970). In addition to glucocorticoids, the hypothalamo-pituitary–thyroid axis is regulated by insulin. Rats that were made insulin deficient by the administration of streptozotocin showed decreases in circulating levels of TSH, T3 and T4 as well as pituitary TSH and hypothalamic TRH content (Gonzalez et al., 1980 and Mitsuma and Nogimori, 1982). Thus, it is unlikely that the hyperinsulinemia observed after acute (this study) and chronic (Baran et al., 2002) ICV NPY but not HS014 administration in pair fed rats could contribute to the differences in effects on thyroid hormone concentrations presently observed, in keeping with the observation that insulin administration is unable to prevent the fasting-induced reduction in thyrotropic axis in rats (Fekete et al., 2006). Taken together, these findings show that acute and chronic ICV NPY administration results in significantly more pronounced and more prolonged

hypercorticosteronemia than MC4 receptor antagonism with HS014, and this difference could contribute to the marked and prolonged inhibition in circulating TSH and free T4 levels that are seen in NPY- relative to HS014-infused rats.

It is intriguing that chronic central infusion of the MC4 receptor antagonist HS014 in rats induced at least as great an increase in white adipose tissue mass as ICV NPY infusion. This is despite the fact that the food intake of HS014- and NPY-infused rats was identical, being yoked in both cohorts at the level of consumption of vehicle-infused control rats to prevent hyperphagia, and infusion of HS014, unlike NPY, did not induce sustained changes in circulating levels of TSH, free T4, corticosterone or insulin (Baran et al., 2002) that could have contributed to fat accumulation over the 6 days. While these hormonal changes may have contributed to the propensity for NPY-infused rats to preferentially deposit excess fat in the mesenteric fat depot, they cannot explain the increased overall adiposity of HS014-infused animals in the absence of hyperphagia. Alternate obesogenic pathways must be at play. For instance, it has been shown that TRH *per se* can increase body temperature, respiration and locomotor activity and stimulate gut functions such as gastric acid secretion, gastro–duodenal blood flow, gastric emptying and gastrointestinal motility in the absence of effects on thyroid hormone concentrations as recently reviewed (Lechan and Fekete, 2006). As such, inhibition of TRH secretion in HS014-infused rats could conceivably contribute to increased adiposity even if circulating thyroid hormone concentrations are unaltered. Additionally, alterations in local tissue conversion of T4 to the more active T3 and the less active reverse T3 by deiodinase enzymes – which are regulated not only by thyroid hormone concentrations but also by sympathetic activity (Bianco et al., 2005) – could also contribute to reduced thyroid function and therefore reduced fuel oxidation in tissues even in the absence of changes in thyroid hormone levels. In keeping with this, we have shown that both NPY and HS014 inhibited protein or mRNA levels of brown adipose tissue UCP-3 (this study) and UCP-1 (Baran et al., 2002), both proteins undergoing major regulation by sympathetic activity and thyroid hormones (Lanni et al., 2003). Additionally, NPY and MC4 receptor antagonism are known to reduce sympathetic output (Bray, 2000), and this is another mechanism via which both NPY and HS014 can alter adiposity independently of changes in food intake or hormonal milieu. Unfortunately it was not possible to measure energy expenditure via indirect calorimetry in this study, which would have facilitated understanding of how chronic central MC4 receptor antagonism increases adiposity in rats despite no difference in energy intake or the circulating concentrations of the hormones under study.

Taken together, these data exemplify the concept that there are multiple distinct mechanisms by which the increases in hypothalamic NPY and AgRP expression that occur during negative energy balance can collectively hinder further weight loss and promote fat accumulation. The increase in central NPY-ergic activity observed during negative energy balance probably contributes more to the associated ongoing decrease in thyrotropic function than increases in central MC4 receptor antagonism. Anti-obesity drugs will undoubtedly need to target multiple systems in order to facilitate effective and continued weight loss during weight loss interventions.

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