Region-specific differences in brain melanocortin receptors in rats of the lean phenotype

Charu Shukla[a], Steven L. Britton[b], Lauren G. Koch[b] and Colleen M. Novak[a]

The brain melanocortin (MC) system is one of numerous overlapping systems regulating energy balance; it consists of peptides including α-melanocyte-stimulating hormone that act through melanocortin receptors (MCRs). Mutations and polymorphisms in MC3R and MC4R have been identified as one of the most common genetic contributors to obesity in human studies. Brain MC3R and MC4R are known to modulate energy expenditure (EE) and food intake, but much less is known regarding brain MC5R. To test the hypothesis that brain MC modulates physical activity (PA) and EE, we compared brain MCR profiles in rats that consistently show high versus low levels of 'spontaneous' daily PA. Compared with low-activity rats, high-activity rats show enhanced mRNA expression of MCRs in the brain, specifically of MC3R in the paraventricular nucleus (PVN), and MC4R and MC5R in the perifornical lateral hypothalamus. Next, we microinjected the MCR agonist melanotan II into the PVN region and measured PA and EE. Intra-PVN melanotan II induced a dose-dependent increase in PA and this effect was greater in high-activity rats compared with low-activity rats. These results indicate region-specific brain MCR expression in the heightened PA seen in association with high endurance capacity and identify promising targets in the brain MC system that may contribute to interindividual variability in energy balance. *NeuroReport* **00:000–000**

NeuroReport 2012, **00**:000–000

Keywords: brain melanocortin system, melanotan II, nonexercise activity thermogenesis, obesity, physical activity

[a]Department of Biological Sciences, Kent State University, Kent, Ohio and [b]Department of Anesthesiology, University of Michigan, Ann Arbor, Michigan, USA

Correspondence to Colleen M. Novak, PhD, Department of Biological Sciences, Kent State University, PO Box 5190, 222 Cunningham Hall, Kent, OH 44242, USA
Tel: +1 330 672 2306; fax: +1 330 670 3713; e-mail: cnovak13@kent.edu

Received 29 March 2012 **accepted** 12 April 2012

Introduction

The increasing prevalence of obesity continues to be a cause of concern worldwide [1]. Recent work has identified the importance of daily physical activity (PA) in both weight management and the risk of cardiovascular disease [1]. The energy expenditure (EE) of daily living, called nonexercise activity thermogenesis (NEAT), has emerged as a crucial factor that accounts for individual differences in body weight, interacting with genetic predisposition [2]. As in humans, animal models of obesity tend to have low levels of PA [2]. In both rats and humans, high intrinsic aerobic capacity is strongly linked to high levels of PA [3]; both these traits may help in the identification of the lean phenotype.

The brain melanocortin (MC) system is one of several overlapping systems regulating energy balance [4]. The prohormone pro-opiomelanocortin (POMC) is cleaved into several bioactive peptides, one of which is α-melanocyte-stimulating hormone (α-MSH). These peptides act as ligands for MC receptor (MCR) subtypes MCR1–5; three of these are found in the adult mammalian brain and mediate a multitude of physiological and behavioral effects [5]. Brain MC3R and MC4R have been shown to modulate EE and food intake, but much less is known about the role of MC5R in the brain [6,7]. Mutations and deficiencies in POMC and MCRs lead to obesity and there is evidence linking this system to PA in humans as well as animal models of obesity [8]. Thus, brain MC circuitry is relevant for a naturally occurring obese phenotype.

In 1996, Koch and Britton [9] initiated the development of rat lines that differed in the intrinsic aerobic treadmill running capacity through two-way (divergent) artificial selection, establishing models for leanness [high-activity rats (HCR)] and obesity [low-activity rats (LCR)]. HCR have consistently higher levels of daily 'spontaneous' PA relative to LCR, independent of differences in body weight or lean mass [10,11]. Our long-term goal is to understand the mechanisms causative of low and high PA as they relate to obesity. Here, we tested the hypothesis that regional MCR expression within the hypothalamus contributes to individual differences in PA in the LCR–HCR model system.

Methods

Quantitative real-time PCR analysis

Nine of each HCR and LCR male rats (generation 21), obtained from the University of Michigan, were individually housed on a 24-h light : dark cycle with ad-libitum access to standard chow (Lab Diet 5001; Lab Diet, Richmond, Indiana, USA) for 28 days [11]. A detailed protocol for obtaining brain micropunches has been described previously [10]. Briefly, brains were taken after rapid decapitation and sectioned using a Hatton apparatus using optic chiasm as the primary landmark. Sections of 100 and 200 were taken, and punches of the

0959-4965 DOI: 10.1097/WNR.0b013e328354f5c1

paraventricular nucleus (PVN) or the perifornical lateral hypothalamus (PeFLH) were extracted using a micropuncher, flash frozen with liquid nitrogen, and stored at −80°C for later processing. Using samples from the arcuate nucleus, PVN, and PeFLH, 20–40 mg of tissue was homogenized for RNA isolation to be estimated by quantitative real-time PCR (Q-PCR). The samples were purified using an RNA purification kit (Ribopure; Ambion Life Technologies, Grand Island, New York, USA) and only samples with optimum RNA integrity numbers were used for further processing. The RNA concentration was measured using NanoDrop (ND-1000; Nanodrop Technologies, Wilmington, Delaware, USA) and ~20–100 ng/μl of isolated RNA was used for cDNA synthesis. Purified total RNA was reverse transcribed using the Applied Biosystems (ABI) Reverse Transcription reagents kit (Carlsbad, California, USA), using the standard suggested protocol with random hexamers. Q-PCR was conducted using the Taqman Universal Master Mix (ABI) on the experimental samples using Taqman probes for the genes of interest, using ~20–100 ng of cDNA. The annealing temperature was 60°C with 40 cycles. All samples were run in triplicate on the Stratagene Mx3005P Real-Time PCR System (Agilent, Carlsbad, California, USA). GAPDH was used as control for all assays and the relative expression was calculated using the comparative C_t method ($\Delta\Delta C_t$) method. The HCR mean $\Delta\Delta C_t$ values were used to define 100%, and each animal's data were calculated as a percentage of the mean. Mean and variance values were calculated and unpaired two-tailed *t*-tests were used for analyses. Differences with a *P*-value of less than 0.05 were considered significant.

Physical activity and energy expenditure measurements induced by melanocortin receptor agonist melanotan II

Six HCR and five LCR female rats (generation 25), also obtained from the University of Michigan, were used for this study. After acclimation to our facility, stereotaxic surgeries were performed to implant guide cannulae aimed at PVN [10,12]. Inhaled isoflurane was used for anesthesia and care was taken to minimize the suffering of animals at each stage of the experiment. After surgery, animals were allowed to recover for 1 week, after which a body composition measurement was taken using magnetic resonance spectroscopy with an EchoMRI-700 (Echo Medical Systems, Houston, Texas, USA) to determine fat and lean mass [13]. The rats were then placed in the calorimetry room, where they were allowed to acclimate in their testing cage for 48 h before the start of any experiment. These rats were then microinjected with either a vehicle (aCSF) or the MCR 3, 4, 5 agonist [melanotan (MTII); Phoenix Pharmaceuticals, Burlingame, California, USA]. Three different doses of MTII (5, 10, and 20 pmol in 200 nl) were used to establish a dose–response curve, with each rat receiving each dose in a random order, separated by 48 h between subsequent injections. Using the Oxymax FAST system (Columbus Instruments, Columbus, Ohio, USA), EE (kcal/h) and PA (counts/min) were measured with a temporal resolution of 30 s for a total of 3 h. The first 20 min of data after injection were not included for analysis to account for handling-induced activity and to allow the air exchange to settle.

Placement of the cannula and the potential spread of microinjected compounds were determined at the end of the study by histologically examining the anatomic placement of an injection of India ink (200 nl). Only rats whose dye injection site corresponded to the stereotaxic coordinates (within 250 μm of the PVN) were used for data analysis. One of the LCR rats died during the study of an unrelated cause and we used simulation-based statistical software (NORM) to estimate the missing activity value on the basis of multiple imputations for one of the doses [14]; however, the imputed data point was not included in post-hoc analyses. We used a 4×2 mixed analysis of variance, with a dose of MTII as the within-subjects independent variable and HCR versus LCR as the between-subjects independent variable; the dependent variables (PA and EE) were analyzed separately. We compared each rat's activity with its vehicle value to account for individual differences in activity. To assess the dose range to use in HCR/LCR, the same experimental procedure was followed in six Sprague–Dawley rats targeting PVN or PeFLH. As a result, we were able to identify the optimal dose range and also proceed with fewer total microinjections in HCR/LCR rats by eliminating one dose in the higher range for further experiments.

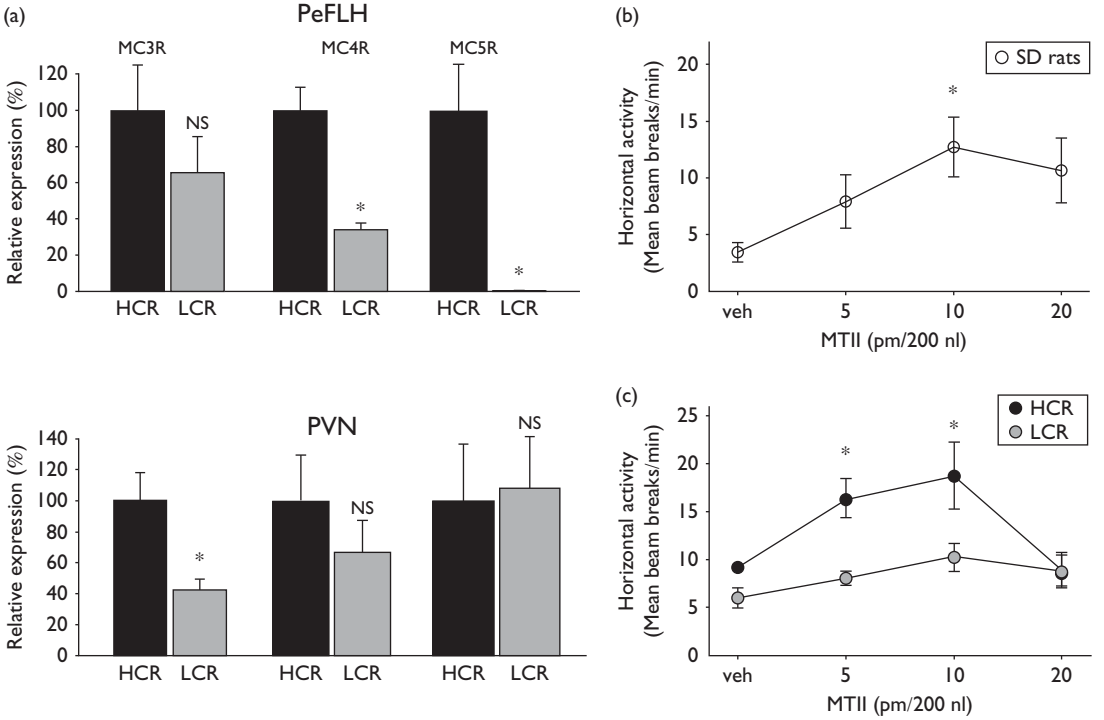
All animal procedures followed were approved by the Institutional Animal Care and Use Committee of Kent State University and were in accordance with the Guide for the Care and Use of Laboratory Animals.

Results

Lean rats have greater site-specific melanocortin receptor mRNA expression

Q-PCR data are shown in Fig. 1a and Table 1. We examined the basal mRNA expression of MC receptors MC3R, MC4R, and MC5R in two brain hypothalamic regions: the PVN and the PeFLH. On comparing HCR with LCR animals, the MC3R mRNA expression level was significantly higher in HCR in the PVN (Fig. 1a). No significant difference was observed in the PeFLH region for this receptor subtype. Both MC4R and MC5R mRNA, however, were significantly higher in HCR in the PeFLH only (Fig. 1a), but not in the PVN. mRNA expression of MC precursor polypeptide POMC, and the processing enzymes prohormone convertase (PC)1 and PC2 in the arcuate nucleus, were not found to be significantly different between LCR and the lean HCR (Table 1). Of the other peptides examined (Table 1), only the expression of brain-derived neurotropic factor (BDNF) in the PeFLH was found to differ significantly between groups (HCR > LCR).

Fig. 1



(a) Quantitative real-time PCR of receptor mRNA levels in target brain regions. Compared with low-activity rats (LCR), high-activity rats (HCR) have greater expression of melanocortin 3, 4, and 5 receptors (MC3R, MC4R, and MC5R) in the paraventricular nucleus (PVN) and the perifornical lateral hypothalamus (PeFLH). MC3R in PeFLH: HCR ($N=8$), LCR ($N=8$); MC4R in PeFLH: HCR ($N=7$), LCR ($N=8$); MC5R in PeFLH: HCR ($N=8$), LCR ($N=8$); MC3R in PVN: HCR ($N=8$), LCR ($N=8$); MC4R in PVN: HCR ($N=7$), LCR ($N=8$); MC5R in PVN: HCR ($N=5$), LCR ($N=8$). (b) Intra-PVN microinjections of melanocortin receptor agonist Melanotan II (MTII) induced physical activity in male Sprague–Dawley (SD) rats ($N=4$); no effect was found after intra-PeFLH injections (data not shown). (c) Intra-PVN MTII-induced activity was significantly greater in HCR ($N=4$) compared with LCR ($N=5$). *$P<0.05$. veh, vehicle.

Melanocortin receptor agonist in specific brain nuclei increases short-term activity and energy expenditure

We measured the activity of Sprague–Dawley rats for a 3-h period after microinjecting the common MCR agonist MTII into the PVN and the PeFLH. We found a short-term increase in home-cage PA with PVN microinjections, which was significant compared with the vehicle aCSF (Fig. 1b). We did not observe a similar increase in the PeFLH region (data not shown). Therefore, we next focused on targeting the PVN region to examine the effect of agonist MTII in HCR and LCR. On comparing HCR and LCR, we found that intra-PVN MTII-induced PA was significantly greater in HCR than LCR (Fig. 1c). EE analysis indicated significant effects of dose, group, and body weight; follow-up analyses of EE (using analysis of covariance) at the 5 pmol/200 nl dose showed greater MTII-induced EE for HCR than LCR.

Discussion

The brain MC system is known to play an important role in satiety and the propensity for obesity. Moreover, evidence that POMC, α-MSH, and MCRs are important in satiety and EE in animal studies has been corroborated

Table 1 Quantitative mRNA measurement of melanocortin receptors in hypothalamic nuclei

Brain region micropunch	Q-PCR probe	*P*-value
ARC	PC1	0.06
	PC2	0.53
	POMC	0.78
	GHSR	0.30
	AgRP	0.10
	NHLH2	0.96
PeFLH	MC3R	0.29
	MC4R	**0.02***
	MC5R	**0.02***
	BDNF	**0.03***
	TRH	0.68
	GHSR	0.15
PVN	**MC3R**	**<0.01***
	MC4R	0.55
	MC5R	0.87
	BDNF	0.13
	TRH	0.86
	GHSR	0.75

AgRP, agouti-related peptide; ARC, arcuate nucleus; BDNF, brain-derived neurotropic factor; GHSR, ghrelin receptors; MC3R, MC4R, MC5R, melanocortin receptor 3, 4, and 5; NHLH2, nescient helix–loop–helix 2 in target brain regions: arcuate nucleus; PC1, PC2, prohormone convertases; PeFLH, perifornical lateral hypothalamus; POMC, pro-opiomelanocortin; PVN, paraventricular nucleus; Q-PCR, quantitative real-time PCR; TRH, thyrotropin-releasing hormone.
*$P<0.05$ (HCR>LCR), highlighted in bold.

by genetic studies in humans that consistently identify MCR point mutations and polymorphisms in human obesity. However, how these genetic differences may possibly manifest to change energetics remains poorly understood. Here, we show for the first time that regional differences in brain MC responses are associated with individual differences in PA, which are linked to obesity propensity or resistance to obesity [6,7,15,16]. These findings may provide an insight into how individuals differ in their brain MC responses, which could in turn make them more or less physically active.

Although POMC expression in the brain is mainly limited to the arcuate nucleus and area prostrema, it is cleaved post-translationally to yield different bioactive compounds that act on MCRs in numerous brain regions to alter several components of energy balance [6,7,15,16]. Heightened POMC results in decreased MCRs and upregulation of α-MSH [4,17], suggesting the possibility that the heightened MCR observed in the high-activity HCR might be secondary to the higher overall MC 'tone' in these rats. However, our data do not support this interpretation. First, it is not likely that differences in activity levels in the rats originate with altered arcuate MC as we found no significant difference in precursor POMC mRNA expression and only a trend in the processing enzyme PC1 (not significantly higher in HCR, $P = 0.06$, Table 1). Second, heightened global MC release would be expected to alter MCRs similarly irrespective of subtype or brain region, whereas we found a region-specific and subtype-specific expression. Altogether, our data suggest that the profile of MCR expression may be intrinsic to the high-activity, high-endurance phenotype.

Moving downstream from the arcuate and area prostema, the action of MCs is limited to three receptors in adult mammalian brain. The receptors MC3R, MC4R, and MC5R are localized to the PVN, PeFLH, and dorsomedial hypothalamus, among other brain regions [7,17]. Our data show that the PVN of the high-activity rats shows higher expression of MC3R, but not of MC4R or MC5R. Moreover, we found that intra-PVN microinjections of MTII increased PA and EE of activity (NEAT), and that the increase in short-term, daytime NEAT was greater in high-activity HCR compared with low-activity LCR. We attribute the heightened EE after intra-PVN MTII to activity EE, but the contribution of intra-PVN MC to brown adipose tissue thermogenesis cannot be ruled out [18]. These results show the possible mechanisms through which individual differences in brain MC contribute to changes in energy balance – specifically EE – that predispose an individual to be lean or obese. This may also suggest that MC3R could contribute to the differences in PA observed between HCR/LCR. Because MTII is not specific for MC3R, however, we cannot rule out the possibility that other MCRs in the PVN contribute to PA and EE. Although a first step, these results can be further defined by the specific localization of function using MCR subtype-specific agonists and antagonists [19,20].

We then examined MC expression in other hypothalamic regions and systems known to be important in regulating PA. Previously, site-specific microinjections of orexin in the orexin cell-body-containing area of the hypothalamus, PeFLH, have been shown to increase PA and EE [10]. We found no differences in the expression of orexin or its receptor that could underlie the differences in PA observed in these rats, however, which led us to examine related parallel brain systems that impact PA. Our data indicate that high-activity HCR show enhanced expression of MC4R and MC5R, but not MC3R, in the PeFLH region. Although several studies have shown the importance of MC4R in obesity and appetite, very little is known about MC5R or its role in the brain in particular. Our results suggest that region-specific differences in MC5R, specifically within the PeFLH, could also contribute toward individual differences in PA. These findings are a good example of the utility of examining the lean phenotype. It has been suggested that standard 'non-obese' animal models might essentially have metabolic syndrome because of housing in a sedentary environment with an unrestricted food supply and limited physical challenges [21]. It is conceivable that MC5R is low or undetectable in the PeFLH of standard rodent models, as it is in the obesity-prone, low-activity LCR (Fig. 1a).

MC is known to interact with several other neuropeptide systems, including orexin [10]. Therefore, we also examined other potential downstream neuropeptide mediators including BDNF and thyrotropin-releasing hormone (TRH), as well as ghrelin receptor (GHSR), all of which are important in these hypothalamic circuits [22]. In the regions examined, BDNF showed a significant difference, being significantly higher in high-activity HCR in the PeFLH (Table 1). BDNF has been described previously as an important component of the hypothalamic pathway that regulates body weight and energy homeostasis [22]. Our results suggest that BDNF may be a critical component in the melanocortinergic brain system regulating spontaneous PA and this system may differ between lean and obesity-prone individuals. Agouti-related peptide is an inverse antagonist of the MCRs and is also known to alter food intake [23]. On examining agouti-related peptide mRNA levels in the arcuate nucleus, we did not detect a difference between HCR/LCR (Table 1). Here, we have focused on hypothalamic circuits, although forebrain reward systems and hindbrain MC are also known to affect energy balance and may potentially differ between high-activity and low-activity individuals [24].

Several lines of evidence highlight the close association between high intrinsic aerobic capacity and high daily PA. In both rats and humans, those with high running endurance are also consistently more physically active [10].

The present study suggests that differences in the brain MC system in these rats, with a regional distribution of MCRs in particular, could underlie individual differences in the tendency to be more or less active. Although MCRs are known to be associated with human obesity, here, we highlight that this effect may partly manifest as differences in PA. While others have shown the relevance of MC3R and MC4R in the regulation of energy balance, satiety, and human obesity, the potential importance of brain MC5R is yet to be considered [7,24,25]. Here, we found that highly specific, differential regional activation of brain MCRs could affect different aspects of energy balance, particularly PA [18].

Acknowledgements

The authors are grateful to Minzhi Zhang for her help with sample collection. The authors acknowledge the expert care of the rat colony provided by Molly Kalahar and Lori Gilligan. The LCR and HCR model can be made available for collaborative study (contact: brittons@umich.edu or lgkoch@umich.edu).

C.M.N. was funded by NIH R01NS055859 and C.S. was funded by AHA 11PRE7320029. The LCR–HCR rat model system was funded by the National Center for Research Resources Grant R24 RR017718 and is currently supported by the Office of Research Infrastructure Programs/OD Grant ROD012098A (to L.G.K. and S.L.B.) from the NIH. S.L.B. was also supported by NIH RO1 DK077200.

Conflicts of interest

There are no conflicts of interest.

References

1 Achike FI, To NH, Wang H, Kwan CY. Obesity, metabolic syndrome, adipocytes and vascular function: a holistic viewpoint. *Clin Exp Pharmacol Physiol* 2011; **38**:1–10.
2 Levine JA, Eberhardt NL, Jensen MD. Role of nonexercise activity thermogenesis in resistance to fat gain in humans. *Science* 1999; **283**: 212–214.
3 Novak CM, Escande C, Gerber SM, Chini EN, Zhang M, Britton SL, *et al.* Endurance capacity, not body size, determines physical activity levels: role of skeletal muscle PEPCK. *PLoS One* 2009; **4**:e5869.
4 Zemel MB, Shi H. Pro-opiomelanocortin (POMC) deficiency and peripheral melanocortins in obesity. *Nutr Rev* 2000; **58**:177–180.
5 Tao YX. The melanocortin-4 receptor: physiology, pharmacology, and pathophysiology. *Endocr Rev* 2010; **31**:506–543.
6 Chen AS, Marsh DJ, Trumbauer ME, Frazier EG, Guan XM, Yu H. Inactivation of the mouse melanocortin-3 receptor results in increased fat mass and reduced lean body mass. *Nat Genet* 2000; **26**:97–102.
7 Mountjoy KG, Wild JM. Melanocortin-4 receptor mRNA expression in the developing autonomic and central nervous systems. *Brain Res Dev Brain Res* 1998; **107**:309–314.
8 Farooqi IS, Keogh JM, Yeo GS, Lank EJ, Cheetham T, O'Rahilly S. Clinical spectrum of obesity and mutations in the melanocortin 4 receptor gene. *N Engl J Med* 2003; **348**:1085–1095.
9 Koch LG, Britton SL. Artificial selection for intrinsic aerobic endurance running capacity in rats. *Physiol Genomics* 2001; **5**:45–52.
10 Novak CM, Escande C, Burghardt PR, Zhang M, Barbosa MT, Chini EN, *et al.* Spontaneous activity, economy of activity, and resistance to diet-induced obesity in rats bred for high intrinsic aerobic capacity. *Horm Behav* 2010; **58**:355–367.
11 Koch LG, Britton SL. Divergent selection for aerobic capacity in rats as a model for complex disease. *Integr Comp Biol* 2005; **45**:405–415.
12 Novak CM, Zhang M, Levine JA. Neuromedin U in the paraventricular and arcuate hypothalamic nuclei increases non-exercise activity thermogenesis. *J Neuroendocrinol* 2006; **18**:594–601.
13 Nixon JP, Zhang M, Wang C, Kuskowski MA, Novak CM, Levine JA, *et al.* Evaluation of a quantitative magnetic resonance imaging system for whole body composition analysis in rodents. *Obesity (Silver Spring)* 2010; **18**:1652–1659.
14 Schafer JL. NORM: Multiple imputation of incomplete multivariate data under a normal model, version 2. 1999.
15 Cauchi S, Stutzmann F, Cavalcanti-Proenca C, Durand E, Pouta A, Hartikainen AL, *et al.* Combined effects of MC4R and FTO common genetic variants on obesity in European general populations. *J Mol Med (Berl)* 2009; **87**:537–546.
16 MacKenzie RG. Obesity-associated mutations in the human melanocortin-4 receptor gene. *Peptides* 2006; **27**:395–403.
17 Pritchard LE, Turnbull AV, White A. Pro-opiomelanocortin processing in the hypothalamus: impact on melanocortin signalling and obesity. *J Endocrinol* 2002; **172**:411–421.
18 Song CK, Jackson RM, Harris RB, Richard D, Bartness TJ. Melanocortin-4 receptor mRNA is expressed in sympathetic nervous system outflow neurons to white adipose tissue. *Am J Physiol Regul Integr Comp Physiol* 2005; **289**:R1467–R1476.
19 Grieco P, Balse PM, Weinberg D, MacNeil T, Hruby VJ. D-Amino acid scan of gamma-melanocyte-stimulating hormone: importance of Trp(8) on human MC3 receptor selectivity. *J Med Chem* 2000; **43**:4998–5002.
20 Grieco P, Cai M, Liu L, Mayorov A, Chandler K, Trivedi D, *et al.* Design and microwave-assisted synthesis of novel macrocyclic peptides active at melanocortin receptors: discovery of potent and selective hMC5R receptor antagonists. *J Med Chem* 2008; **51**:2701–2707.
21 Martin B, Ji S, Maudsley S, Mattson MP. 'Control' laboratory rodents are metabolically morbid: why it matters. *Proc Natl Acad Sci USA* 2010; **107**:6127–6133.
22 Wang C, Bomberg E, Billington CJ, Levine AS, Kotz CM. Brain-derived neurotrophic factor (BDNF) in the hypothalamic ventromedial nucleus increases energy expenditure. *Brain Res* 2010; **1336**:66–77.
23 Stutz AM, Morrison CD, Argyropoulos G. The agouti-related protein and its role in energy homeostasis. *Peptides* 2005; **26**:1771–1781.
24 Bromberg-Martin ES, Hikosaka O. Midbrain dopamine neurons signal preference for advance information about upcoming rewards. *Neuron* 2009; **63**:119–126.
25 Butler AA, Kesterson RA, Khong K, Cullen MJ, Pelleymounter MA, Dekoning J, *et al.* A unique metabolic syndrome causes obesity in the melanocortin-3 receptor-deficient mouse. *Endocrinology* 2000; **141**:3518–3521.