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a-MSH enhances activity-based anorexia

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Abstract

Activity-based anorexia (ABA) is considered an animal model of anorexia nervosa (AN). In ABA, scheduled feeding in combination with voluntary access to running wheels, results in hyperactivity, hypophagia, body weight loss and activation of the HPA axis. Since stimulation of the melanocortin (MC) system has similar effects, this system is a candidate system involved in ABA. Here it is shown that chronic a-MSH treatment enhances ABA by increasing running wheel activity (RWA), decreasing food intake and increasing HPA axis activation.

Keywords: Hyperactivity; Food restriction; Melanocortins; Anorexia nervosa

1. Introduction

Anorexia nervosa (AN) is a psychiatric disorder often characterized by extreme hypophagia, severe body weight loss and hyperactivity [8,30]. AN mainly affects teenage girls and has the highest mortality rate among psychiatric disorders [44]. By mimicking aspects of AN in rats, more insight can be obtained on physiological aspects underlying the human disease.

Activity-based anorexia (ABA) is considered an animal model of AN. The phenomenon of ABA was first described in the early fifties of the last century [21,40]. ABA (also known as activity-stress or activity-anorexia) is based upon the combination of scheduled feeding and voluntary wheel running. In ABA, scheduled feeding results in increased running wheel activity (RWA), decreased food intake (as compared to food-restricted controls), extensive body weight loss (>20%) and increased activity of the hypothalamus-pituitary-adrenal (HPA) axis [40]. Not only total RWA increases, but the distribution of activity throughout the day changes as well. Rats develop food-anticipatory activity (FAA), which in general takes place 3–4 h preceding food intake [35]. ABA rats also show hypothermia, loss of estrous cycle, stomach ulceration and will eventually die [6,38].

A candidate system possibly underlying the phenomenon ABA is the melanocortin (MC) system. The brain MC system comprises of (a) proopiomelanocortin (POMC) neurons in the arcuate nucleus (ARC) and nucleus of the solitary tract [9,17]; (b) agouti-related protein (AGRP) neurons in the ARC [4,20] and (c) melanocortin 3 and 4 receptors (MCRs), which are widely distributed throughout the brain [16,36,39]. Stimulation of the MC system by intracerebroventricular (icv) infusion of a-melanocyte-stimulating hormone (a-MSH) or its analogs results in hypophagia [13,46]. a-MSH infusion raises metabolic rate, increases sympathetic nerve traffic to brown adipose tissue and stimulates lipolysis in ob/ob mice and wildtype rats [15,23]. Furthermore, a-MSH treatment influences HPA axis activity by increasing adrenocorticotropin (ACTH) and corticosterone release in rats [10,48]. These effects can be antagonized by treatment with the competitive MC antagonist SHU9119 [1,13,41]. Furthermore, the inverse agonist $AGRP_{(83–132)}$ increases food intake, decreases oxygen consumption and reduces capacity of brown adipose tissue to expend energy [19,32,43].

Now that the role of the MC system in obesity is widely accepted [7,26,31,45], it was hypothesized that inadequate suppression of the MC system may contribute to development or maintenance of anorexia. Given that stimulation of

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MC activity decreases food intake, increases energy expenditure and stimulates the HPA axis (phenomena which also occur in ABA), a hyperactive MC system possibly underlies ABA. It has recently been reported that during ABA, MCR binding sites are increased in the ventromedial hypothalamus (VMH), indeed suggesting hyperactivity of the MC system [27]. Furthermore, it was shown that suppression of MC activity by chronic icv infusion of $AGRP_{(83–132)}$ increases survival of rats in the ABA model [27]. In the present study, it was investigated whether stimulation of melanocortinergic activity by chronic icv infusion of a-MSH enhances development of ABA.

2. Materials and methods

2.1. Rats

Female outbred Wistar WU rats ($n = 33$, Harlan, Horst, The Netherlands) weighing 160 g upon arrival were individually housed in a temperature and humidity controlled room ($21 \pm 2\,^{\circ}C$) under a 12:12 h dark:light cycle (ZT12 = lights off). The ethical committee on use and care of animals of Utrecht University approved all described procedures. For ethical reasons, rats eating less than 4 g chow on 2 consecutive days were removed from the experiment before day 6 and were excluded from analysis.

2.2. Drugs

a-MSH (24 μg/day) (Bachem, Bubendorf, Switzerland) was dissolved in sterile isotonic saline and was chronically (continuous during 4.5 days) infused (12 μl/day) into the lateral ventricle using osmotic minipumps (Alzet, model 1007D, DURECT Corporation, Cupertino, CA, USA).

2.3. Surgical procedures

Rats were anaesthetized by fentanyl/fluanisone (Hypnorm®, Janssen Pharmaceutica, Beerse, Belgium, 0.1 ml/100 g im) and midazolam (Dormicum®, Hoffman-LaRoche, Mijdrecht, The Netherlands, 0.05 ml/100 g i.p.) for icv surgery. The head was shaven and the skull was exposed by a midline incision. A brain infusion cannula (Alzet, brain infusion kit 3–5 mm) was placed into the lateral ventricle 1 mm lateral, 1 mm posterior from bregma and fixed in place with two small screws and dental cement. The cannula was connected by tubing (filled with vehicle) to an osmotic minipump containing vehicle or a-MSH. Minipumps were subcutaneously placed into the flank region of the rat after overnight incubation at 37 °C. The polyvinylchloride tubing between the minipump and the cannula was filled with vehicle and had a fixed length (8 cm). Considering tubing characteristics (i.d. 0.69 mm, o.d. 1.4.mm) and pump rate (0.5 μl/h), it was calculated that the ligand from the pump entered the brain ± 60 h following surgery. After surgery, rats were treated with buprenorphin (Temgesic®, Schering-Plough, Maarssen, The Netherlands, 0.05 ml/100 g s.c.) and saline (1 ml s.c.).

2.4. Experimental set-up

Since it was hypothesized that a-MSH treatment would decrease food intake in ABA rats, resulting in (an even more) rapid body weight loss and early collapse in ABA, it was decided to use a 2 h feeding schedule, which normally results in a slower development of ABA [11]. Thus, the effects of a-MSH treatment in 2 h-fed rats were compared to the effects of vehicle treatment in 2 h-fed rats and 1 h-fed rats (normal ABA procedure). After 1 week acclimatization to the animal facility, rats were individually housed in cages with running wheels for a training period of 10 days (from days −10 to 0). RWA was continuously registered using a Cage Registration Program (Dep. Biomedical Engineering, UMC Utrecht, The Netherlands). At the end of day −1, rats were divided into three groups matched for 4-day RWA (average day −4: day −1: 7655.0 ± 589.2 revolutions) and body weight (average day −1: 195.2 ± 1.9 g). In all rats, icv cannulae and osmotic minipumps were implanted as indicated above. After surgery (day 0, ZT12) food was removed from all cages. The next days, rats had 1 or 2 h(s) access to food during the first hour(s) of the dark phase, while water was continuously available. Body weight, food intake and RWA were measured daily. At the end of day 6 (ZT11) rats were decapitated. Trunk blood was collected in lithium-heparin (Sarstedt, Nümbrecht, Germany) containing tubes after adding 83 μmol EDTA and 1 mg aprotonin. Plasma was separated and frozen at −20 °C. Brains were rapidly removed, quickly frozen in cold (−35 °C) isopentane and stored at −80 °C. Adrenal glands were isolated and weighed.

2.5. In situ hybridization

Cryosections (coronal, 20 μm) of the hypothalamus of 2 h-fed vehicle and a-MSH treated rats were sliced using a cryostat (Leica, Rijswijk, The Netherlands) and thaw-mounted onto RNAse free Superfrost slides (Menzel, Germany). The slides were stored at −80 °C until processing for in situ hybridization. All cryostat sections were concurrently prepared for hybridization and used in the same assay for each probe. Sections were fixed in 4% paraformaldehyde (PFA) in phosphate-buffered-saline (PBS) for 10 min, washed in PBS, pretreated with 0.25% acetic anhydride in 0.1 M triethanolamine, washed again in PBS and dehydrated in graded ethanol followed by 100% chloroform and 100% ethanol. ^{33}P-labeled antisense RNA probes were made using a 350 bp rat POMC cDNA fragment [27], a 286 bp rat NPY cDNA fragment [12] and a 396 bp mouse AGRP cDNA fragment [27]. The sections were hybridized overnight at 72 °C with 1×10^6 cpm probe in buffer containing 50% deionized formamide, 2× standard saline citrate (SSC), 10% dextrane sulphate, 1× Denhardt's solution, 5 mM EDTA

and 10 mM phosphate buffer, after 5 min heating at 80 °C. After hybridization, the sections were washed in 5× SSC (short, 72 °C) and 0.2× SSC (2 h, 72 °C) and dehydrated in graded ethanol with 3 M ammoniumacetate. Sections were exposed to X-ray films (Kodak Bio-Max MR) for 5 days. The films were developed and film absorbance values (converted to radioactivity concentrations using a standard curve) reflecting POMC, AGRP and NPY expression were semi-quantitatively analyzed using the Microcomputer Imaging Device (MCID) (Imaging Research Inc., St. Catharine's, Ont., Canada).

2.6. Radioimmunoassay

Plasma levels of corticosterone and ACTH were analyzed by radioimmunoassay (RIA). Corticosterone was measured using a commercially available rat corticosterone RIA kit (ICN Biochemicals, Costa Mesa, CA, USA). ACTH was measured using a specific rabbit antiserum directed to the midportion of ACTH kindly provided by Dr. G.B. Makara (Budapest, Hungary). Synthetic human $ACTH_{(1–39)}$ (Peninsula Laboratories, Belmont, CA, USA) was labeled with ^{125}I and used as a tracer [37].

2.7. Data analysis

All data are presented as mean ± standard error. Data were analyzed using SPSS 11.5 for Windows and were controlled for normality and homogeneity. Four rats (2× 2 h a-MSH-treated, 2× 1 h vehicle-treated) were removed from the experiment before day 6 (due to low food intake) and were excluded from all analysis. For all measurements baseline levels were not significantly different between groups. RWA, relative body weight and food intake were analyzed by GLM repeated measures analysis using Huynh Feldt correction for Mauchlys sphericity effects followed by ANOVA using Bonferroni as a post hoc test. Time (days) was used as a within-subjects factor and condition (2 h-fed + vehicle, 2 h-fed + a-MSH or 1 h-fed + vehicle) was used as a between-subjects factor. Body weight loss, cumulative food intake (days 1–6) and HPA axis activation were analyzed by ANOVA using Bonferroni as post hoc test. Furthermore, cumulative food intake (days 3–6) and RWA (days 4–5) were more thoroughly analyzed during the period of ligand infusion by ANOVA using Bonferroni as post hoc test. ISH data were analyzed by *t*-tests. Differences were considered significant at $P < 0.05$.

3. Results

Running wheel activity in the light phase was significantly influenced by condition over time (day: $F(6,156) = 30.60$, $P < 0.001$, day *x* condition: $F(12,156) = 4.05$, $P < 0.001$), whereas dark phase RWA (day: $F(6,156) = 19.69$, $P < 0.001$, day *x* condition: $F(12,156) = 0.31$, n.s.) and total daily RWA (day: $F(6,156) = 30.00$, $P < 0.001$, day *x* condition: $F(12,156) = 1.06$, n.s.) were not significantly affected (Fig. 1A). a-MSH-treated rats showed increased RWA during the light phase as compared to 2 h-fed vehicle-treated rats, but not as compared to 1 h-fed controls. More thorough analysis of RWA at day 4 and 5 (in the middle of the period of a-MSH infusion) revealed that total light phase activity ($F(2,28) = 5.89$, $P = 0.01$) but not total dark phase activity ($F(2,28) = 0.16$, n.s.) was increased in a-MSH-treated rats as compared to 2 h-fed vehicle-treated rats ($P = 0.01$), but not as compared to 1-hr fed controls (Fig. 1B). a-MSH treated rats developed light phase RWA earlier than 2 h-fed controls

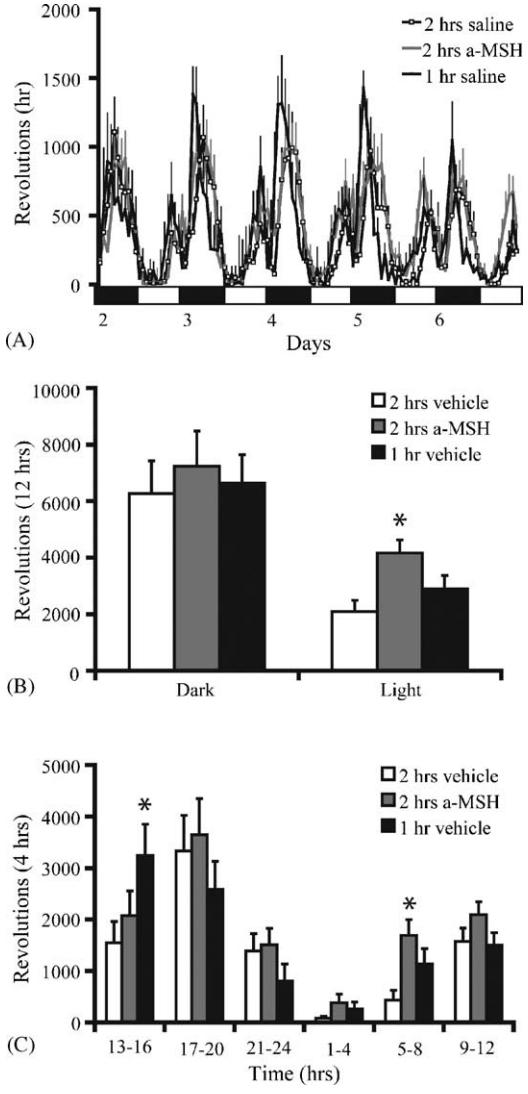

Fig. 1. Running wheel activity in vehicle-treated and a-MSH-treated rats exposed to the ABA model. Revolutions per hour at days 2–6 in 2 h-fed vehicle-treated ($n = 12$), 2 h-fed a-melanocyte-stimulating hormone (a-MSH) treated ($n = 12$) and 1 h-fed vehicle-treated ($n = 5$) rats exposed to the activity-based anorexia (ABA) model. a-MSH infusion entered the brain in the light phase of day 2 (A). Revolutions per dark/light phase on days 4 and 5 (B) and per 4-h periods on days 4 and 5 (C) of 2 h-fed vehicle-treated ($n = 12$), 1 h-fed vehicle-treated ($n = 5$) and 2 h-fed a-MSH-treated rats ($n = 12$) ABA rats. Data indicate mean values ± S.E. ANOVA Bonferroni, $^{*}P < 0.05$ vs. 2 h-fed vehicle.

Table 1
Characteristics of ABA in vehicle-treated and a-MSH-treated rats

Condition	Final body weight (%)	4/6 day food intake (g)	ACTH (pg/ml)	Corticosterone (μg/dl)	Adrenal weight (%)
2-h + vehicle ($n = 12$) (a)	80.8 ± 1.2[b,c]	40.0 ± 1.6[b,c]; 50.2 ± 1.8[b,c]	162.6 ± 17.1[b]	27.2 ± 3.2	0.030 ± 0.001[b]
2-h + a-MSH ($n = 12$) (b)	72.8 ± 1.4[a]	30.0 ± 1.3[a,c]; 39.1 ± 1.7[a,c]	640.8 ± 105.8[a,c]	35.6 ± 3.3	0.034 ± 0.001[a]
1 h + vehicle ($n = 5$) (c)	70.7 ± 2.7[a]	22.9 ± 0.2[a,b]; 28.2 ± 0.4[a,b]	237.4 ± 50.1[b]	27.1 ± 4.2	0.031 ± 0.004

Two hour-fed rats treated with vehicle (a) or a-melanocyte-stimulating hormone (a-MSH) (b) and 1 h-fed-vehicle-treated controls (c) had access to food during the first hour(s) of the dark phase starting on day 1. a-MSH entered the brain 60 h following surgery, in the light phase of day 2. Final body weight (%day −1), 4-day food intake (=a-MSH infusion, days 3–6), 6-day food intake (days 1–6), plasma ACTH, plasma corticosterone and relative adrenal glands weight (% bw day 6) were examined. Data indicate mean values ± S.E. Superscript letters (a–c) indicate values different from condition, ANOVA Bonferroni, $P < 0.05$.

(Fig. 1C). Although 1 h-fed controls showed higher levels of RWA (dark and light) than 2 h-fed vehicle-treated rats, this difference was not significant.

Relative body weight was significantly different among conditions over time (day: $F(6,156) = 358.97$, $P < 0.001$, day *x* condition: $F(12,156) = 11.06$, $P < 0.001$). a-MSH-treated rats and 1 h-fed vehicle treated rats lost more body weight than 2 h-fed controls ($F(2,28) = 11.51$, $P < 0.001$) (both $P < 0.001$).

Statistical analysis also revealed an interaction of condition and time on food intake (day: $F(5,130) = 81.06$, $P < 0.001$, day *x* condition: $F(10,130) = 3.27$, $P < 0.001$). Cumulative food intake during 4 days of a-MSH infusion was significantly different among all conditions ($F(2,28) = 28.86$, $P < 0.001$). a-MSH-treated rats and 1 h-fed controls decreased food intake as compared to 2 h-fed controls (both $P < 0.001$), however 2 h-fed a-MSH-treated rats consumed more than 1 h-fed controls ($P = 0.02$).

Plasma ACTH levels were significantly influenced by condition ($F(2,28) = 12.32$, $P < 0.001$). a-MSH-treated rats had elevated plasma ACTH levels as compared to both control groups (both $P < 0.001$). The weight of adrenal glands was significantly ($F(2,28) = 4.25$, $P = 0.03$) increased in a-MSH-treated rats as compared to 2 h-fed controls, also when corrected for body weight ($P = 0.03$). Plasma corticosterone levels were not significantly different among condition ($F(2,28) = 2.10$, n.s.), which was probably due to time of plasma collection (near the peak of diurnal corticosterone release) (Table 1).

By in situ hybridization it was shown that AGRP and NPY expression levels were significantly increased in a-MSH-treated rats as compared to 2 h-fed vehicle-treated rats, whereas POMC expression was non significantly decreased (AGRP: $t(8) = -4.74$, $P < 0.001$, NPY: $t(7) = -4.44$, $P = 0.003$, POMC: $t(7) = 1.24$, n.s.) (Fig. 2).

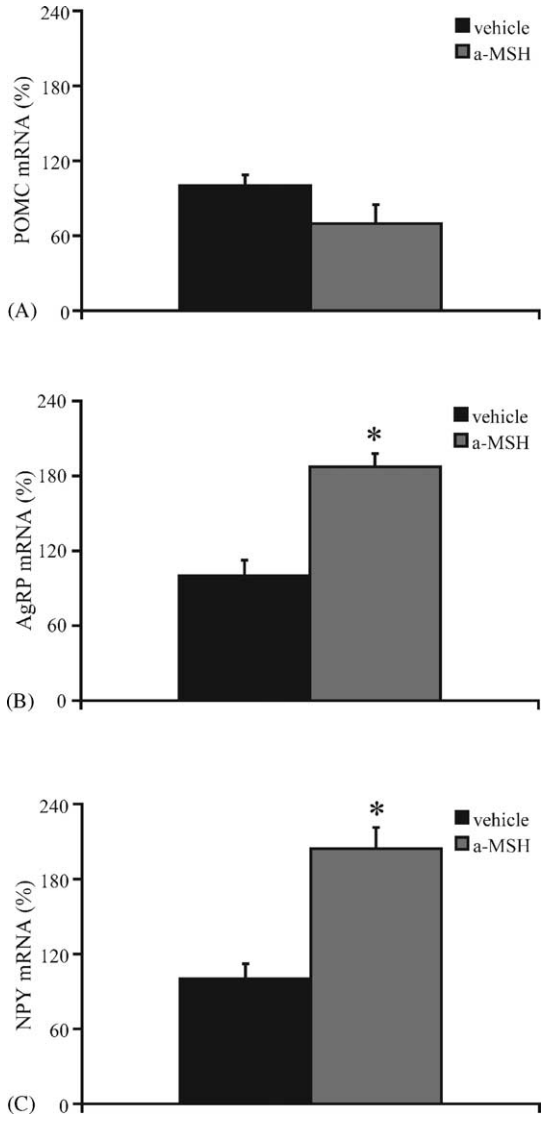

Fig. 2. POMC, AGRP and NPY gene expression in vehicle-treated and a-MSH-treated rats following exposure to the ABA model. Proopiomelanocortin (POMC), agouti-related protein (AGRP) and Neuropeptide Y (NPY) expression in the arcuate nucleus was semi-quantitatively measured by radioactive in situ hybridization in rats exposed to activity-based anorexia (ABA) (2 h feeding) for 1 week and treated with vehicle ($n = 5$) or a-melanocyte-stimulating hormone (a-MSH) ($n = 5$). Data is expressed as percentage of 2 h-fed vehicle treated ABA rats (mean ± S.E.). *t*-test; $P < 0.05$.

4. Discussion

a-MSH treatment in 2 h-fed rats increased RWA in the light phase, decreased food intake, decreased body weight and increased activity of the HPA axis as compared to 2 h-fed vehicle-treated rats. Therefore, it is concluded that chronic a-MSH infusion enhances ABA. a-MSH-treated rats responded

similar to 1 h-fed vehicle-treated rats regarding RWA and body weight loss. However, food intake and plasma ACTH levels were significantly different between these two groups.

ABA rats still responded to a-MSH treatment by decreasing food intake. Increased AGRP and NPY expression reflected their negative energy balance following a-MSH treatment. This is noteworthy since it was reported before that the anorectic efficacy of a-MSH wanes with chronic administration [34]. Moreover, it was recently shown that scheduled feeding attenuates the anorectic effects of MTII, an a-MSH analog, which might be explained by anticipatory physiological effects arising during scheduled feeding [5]. Apparently ABA rats are in a unique state and still respond to a-MSH treatment regardless of their catabolic state.

All ABA rats developed RWA prior to food access. This food-anticipatory activity seems to have compulsive characteristics [2]. In our experimental setting, FAA took place at the end of the light phase (which is normally the inactive period of the day). a-MSH treatment did not influence total daily RWA or dark phase RWA, but specifically increased light phase activity (thus FAA) in this study. In a-MSH treated rats, FAA was extended to earlier parts of the light phase (7 h before lights off) (Fig. 1).

It has been reported before that increasing the duration of feeding (1 h versus 2 h) delays the development of ABA [11]. In the present experiment, it was shown that food intake and body weight were significantly decreased in 1 h-fed rats as compared to 2 h-fed vehicle-treated rats, confirming that the length of the feeding period is a crucial factor in development of ABA. RWA was not significantly different between 1 h-fed vehicle-treated rats and 2 h-fed vehicle-treated rats, which might be explained by large individual differences in RWA in both groups.

It has been described before that a-MSH treatment influences HPA axis activity in ad libitum fed rats [10,48]. During ABA, the HPA axis is activated resulting in increased levels of corticosterone or ACTH and enlarged adrenal glands [6,27]. Here it was shown that a-MSH treatment further increased HPA axis activity, thereby possibly contributing to a rapid development of ABA. Alternatively, increased HPA axis activity following a-MSH treatment might reflect additional body weight loss induced by a-MSH. However, there were no body weight differences between 1 h-fed vehicle-treated rats and 2 h-fed a-MSH-treated rats, but their plasma ACTH levels were significantly different, suggesting a direct influence of a-MSH treatment on HPA axis activity.

Previously it was shown that $AGRP_{(83–132)}$ (chronic icv 5.6 μg/day) treatment increased survival in ABA rats [27]. In the same experimental set-up as used in this study, $AGRP_{(83–132)}$ treatment increased food intake in rats on a 1 h feeding schedule and partly prevented the starvation-induced decrease in body temperature, resulting in increased survival. Whereas a-MSH treatment increased FAA, $AGRP_{(83–132)}$ treatment did not influence FAA or total RWA. Furthermore, $AGRP_{(83–132)}$ treatment did not change HPA axis activity in ABA, similar to results from another study reporting that chronic $AGRP_{(83–132)}$ treatment in ad libitum fed and food-restricted rats does not influence plasma corticosterone levels or adrenal gland weight [42]. Hence, it appears that stimulation of MCRs by a-MSH and suppression of MCRs by $AGRP_{(83–132)}$ influence ABA, although not all parameters of ABA were influenced by both treatments.

Further evidence for a role of the MC system in ABA was reported before by Kas et al. [27], who showed that ^{125}I-NDP-MSH binding sites are increased in the VMH, but not in the Habenular nucleus (a region not involved in feeding behavior) after 1 week exposure to ABA. Since the VMH itself has only minor a-MSH and AGRP neuronal innervation, increased ^{125}I-NDP-MSH binding sites are most likely an effect of constitutive activity of the MCRs [4]. Increased density of MCRs during food restriction has been shown before [22] and suggests increased activity of the MC system in ABA, which seems paradoxical in a situation of negative energy balance. Exogenously applied a-MSH in the present study may directly activate MCRs in the VMH, which may contribute to the development of ABA.

Hyperactivity of the MC system can also be generated by increased expression of POMC, increased processing and release of a-MSH or decreased expression and release of AGRP. However, ARC POMC gene expression is decreased (although not significantly different) and ARC AGRP expression is increased after 1 week exposure to ABA, thus, counteracting the increased MCRs response [27,49]. Since MCR binding studies and gene expression studies were all performed after 1 week exposure to ABA, it would be interesting to examine changes in gene expression and MCR binding early during development of ABA.

ABA rats display similar characteristics as AN patients; e.g. starvation, hyperactivity, considerable body weight loss and increased activity of the HPA axis. Changes in the MC system have also been reported in AN patients. Levels of POMC-related peptides in cerebrospinal fluid are decreased in ill and short-term recovered patients, while normalized in long-term recovered patients [28]. In addition, AN patients show increased levels of plasma cortisol and CRH, indicating increased activity of the HPA axis [18,25,29]. Recently, the presence of auto-antibodies against the MC system in plasma of AN patients was described, which could possibly interfere with MC signaling and contribute to pathophysiology in AN patients [14]. Polymorphisms in the MC4R gene (Val103Ile, Ile251Leu) and AGRP gene (Ala67Thr) have also been described in populations of AN patients. Although the occurrence of the MC4R polymorphisms was not different from a control population [24], the AGRP polymorphism occurred more frequently in a population of AN patients as compared to controls [47] and was associated with low body weight in two other populations [3,33].

In conclusion, chronic a-MSH treatment enhances ABA by decreasing food intake and increasing FAA and HPA axis activation. Together with previous findings showing that $AGRP_{(83–132)}$ treatment inhibits ABA and evidence for increased MCR binding in the VMH of ABA rats, these

results suggest that the MC system plays an important role in ABA.

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References

[1] Adage T, Scheurink AJ, de Boer SF, de Vries K, Konsman JP, Kuipers F, et al. Hypothalamic, metabolic, and behavioral responses to pharmacological inhibition of CNS melanocortin signaling in rats. J Neurosci 2001;21:3639–45.
[2] Altemus M, Glowa JR, Galliven E, Leong YM, Murphy DL. Effects of serotonergic agents on food-restriction-induced hyperactivity. Pharmacol Biochem Behav 1996;53:123–31.
[3] Argyropoulos G, Rankinen T, Neufeld DR, Rice T, Province MA, Leon AS, et al. A polymorphism in the human agouti-related protein is associated with late-onset obesity. J Clin Endocrinol Metab 2002;87:4198–202.
[4] Bagnol D, Lu XY, Kaelin CB, Day HE, Ollmann M, Gantz I, et al. Anatomy of an endogenous antagonist: relationship between Agouti-related protein and proopiomelanocortin in brain. J Neurosci 1999;19. RC26.
[5] Benoit SC, Clegg DJ, Barrera JG, Seeley RJ, Woods SC. Learned meal initiation attenuates the anorexic effects of the melanocortin agonist MTII. Diabetes 2003;52:2684–8.
[6] Burden VR, White BD, Dean RG, Martin RJ. Activity of the hypothalamic-pituitary-adrenal axis is elevated in rats with activity-based anorexia. J Nutr 1993;123:1217–25.
[7] 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;141:3518–21.
[8] Casper RC, Schoeller DA, Kushner R, Hnilicka J, Gold ST. Total daily energy expenditure and activity level in anorexia nervosa. Am J Clin Nutr 1991;53:1143–50.
[9] Castro MG, Morrison E. Post-translational processing of proopiomelanocortin in the pituitary and in the brain. Crit Rev Neurobiol 1997;11:35–57.
[10] Dhillo WS, Small CJ, Seal LJ, Kim MS, Stanley SA, Murphy KG, et al. The hypothalamic melanocortin system stimulates the hypothalamo-pituitary-adrenal axis in vitro and in vivo in male rats. Neuroendocrinology 2002;75:209–16.
[11] Dwyer DM, Boakes RA. Activity-based anorexia in rats as failure to adapt to a feeding schedule. Behav Neurosci 1997;111:195–205.
[12] Ericsson A, Schalling M, McIntyre KR, Lundberg JM, Larhammar D, Seroogy K, et al. Detection of neuropeptide Y and its mRNA in megakaryocytes: enhanced levels in certain autoimmune mice. Proc Natl Acad Sci USA 1987;84:5585–9.
[13] Fan W, Boston BA, Kesterson RA, Hruby VJ, Cone RD. Role of melanocortinergic neurons in feeding and the agouti obesity syndrome. Nature 1997;85:65–168.
[14] Fetissov SO, Hallman J, Oreland L, Af KB, Grenback E, Hulting AL, et al. Autoantibodies against alpha-MSH, ACTH, and LHRH in anorexia and bulimia nervosa patients. Proc Natl Acad Sci USA 2002;99:17155–60.
[15] Forbes S, Bui S, Robinson BR, Hochgeschwender U, Brennan MB. Integrated control of appetite and fat metabolism by the leptin-proopiomelanocortin pathway. Proc Natl Acad Sci USA 2001;98:4233–7.
[16] Gantz I, Miwa H, Konda Y, Shimoto Y, Tashiro T, Watson SJ, et al. Molecular cloning, expression, and gene localization of a fourth melanocortin receptor. J Biol Chem 1993;268:15174–9.
[17] Gee CE, Chen CL, Roberts JL, Thompson R, Watson SJ. Identification of proopiomelanocortin neurones in rat hypothalamus by in situ cDNA-mRNA hybridization. Nature 1983;306:374–6.
[18] Gold PW, Gwirtsman H, Avgerinos PC, Nieman LK, Gallucci WT, Kaye W, et al. Abnormal hypothalamic-pituitary-adrenal function in anorexia nervosa. Pathophysiologic mechanisms in underweight and weight-corrected patients. N Engl J Med 1986;314:1335–42.
[19] Hagan MM, Rushing PA, Pritchard LM, Schwartz MW, Strack AM, Van der Ploeg LH, et al. Long-term orexigenic effects of AgRP-(83–132) involve mechanisms other than melanocortin receptor blockade. Am J Physiol Regul Integr Comp Physiol 2000;279:R47–52.
[20] Hahn TM, Breininger JF, Baskin DG, Schwartz MW. Coexpression of Agrp and NPY in fasting-activated hypothalamic neurons. Nat Neurosci 1998;1:271–2.
[21] Hall JF, Hanford PV. Activity as a function of a restricted feeding schedule. J Comp Physiol Psychol 1954;47:362–3.
[22] Harrold JA, Widdowson PS, Williams G. Altered energy balance causes selective changes in melanocortin-4(MC4-R), but not melanocortin-3 (MC3-R), receptors in specific hypothalamic regions: further evidence that activation of MC4-R is a physiological inhibitor of feeding. Diabetes 1999;48:267–71.
[23] Haynes WG, Morgan DA, Djalali A, Sivitz WI, Mark AL. Interactions between the melanocortin system and leptin in control of sympathetic nerve traffic. Hypertension 1999;33:542–7.
[24] Hinney A, Schmidt A, Nottebom K, Heibult O, Becker I, Ziegler A, et al. Several mutations in the melanocortin-4 receptor gene including a nonsense and a frameshift mutation associated with dominantly inherited obesity in humans. J Clin Endocrinol Metab 1999;84:1483–6.
[25] Hotta M, Shibasaki T, Masuda A, Imaki T, Demura H, Ling N, et al. The responses of plasma adrenocorticotropin and cortisol to corticotropin-releasing hormone (CRH) and cerebrospinal fluid immunoreactive CRH in anorexia nervosa patients. J Clin Endocrinol Metab 1986;62:319–24.
[26] Huszar D, Lynch CA, Fairchild-Huntress V, Dunmore JH, Fang Q, Berkemeier LR, et al. Targeted disruption of the melanocortin-4 receptor results in obesity in mice. Cell 1997;88:131–41.
[27] Kas MJ, van Dijk G, Scheurink AJ, Adan RA. Agouti-related protein prevents self-starvation. Mol Psychiatry 2003;8:235–40.
[28] Kaye WH, Berrettini WH, Gwirtsman HE, Chretien M, Gold PW, George DT, et al. Reduced cerebrospinal fluid levels of immunoreactive pro-opiomelanocortin related peptides (including beta-endorphin) in anorexia nervosa. Life Sci 1987;41:2147–55.
[29] Kaye WH, Gwirtsman HE, George DT, Ebert MH, Jimerson DC, Tomai TP, et al. Elevated cerebrospinal fluid levels of immunoreactive corticotropin-releasing hormone in anorexia nervosa: relation to state of nutrition, adrenal function, and intensity of depression. J Clin Endocrinol Metab 1987;64:203–8.
[30] Kron L, Katz JL, Gorzynski G, Weiner H. Hyperactivity in anorexia nervosa: a fundamental clinical feature. Compr Psychiatry 1978;19:433–40.
[31] Krude H, Biebermann H, Luck W, Horn R, Brabant G, Gruters A. Severe early-onset obesity, adrenal insufficiency and red hair pigmentation caused by POMC mutations in humans. Nat Genet 1998;19:155–7.
[32] Lu D, Willard D, Patel IR, Kadwell S, Overton L, Kost T, et al. Agouti protein is an antagonist of the melanocyte-stimulating-hormone receptor. Nature 1994;371:799–802.

[33] Marks DL, Boucher N, Lanouette CM, Perusse L, Brookhart G, Comuzzie AG, et al. Ala67Thr polymorphism in the Agouti-related peptide gene is associated with inherited leanness in humans. Am J Med Genet 2004;126A:267–71.
[34] McMinn JE, Wilkinson CW, Havel PJ, Woods SC, Schwartz MW. Effect of intracerebroventricular alpha-MSH on food intake, adiposity, c-Fos induction, and neuropeptide expression. Am J Physiol Regul Integr Comp Physiol 2000;279:R695–703.
[35] Mistlberger RE. Circadian food-anticipatory activity: formal models and physiological mechanisms. Neurosci Biobehav Rev 1994;18:171–95.
[36] Mountjoy KG, Mortrud MT, Low MJ, Simerly RB, Cone RD. Localization of the melanocortin-4 receptor (MC4-R) in neuroendocrine and autonomic control circuits in the brain. Mol Endocrinol 1994;8:1298–308.
[37] Nijsen MJ, Croiset G, Stam R, Bruijnzeel A, Diamant M, De Wied D, et al. The role of the CRH type 1 receptor in autonomic responses to corticotropin- releasing hormone in the rat. Neuropsychopharmacology 2000;22:388–99.
[38] Pare WP, Vincent GP, Isom KE, Reeves JM. Sex differences and incidence of activity-stress ulcers in the rat. Psychol Rep 1978;43:591–4.
[39] Roselli-Rehfuss L, Mountjoy KG, Robbins LS, Mortrud MT, Low MJ, Tatro JB, et al. Identification of a receptor for gamma melanotropin and other proopiomelanocortin peptides in the hypothalamus and limbic system. Proc Natl Acad Sci USA 1993;90:8856–60.
[40] Routtenberg A, Kuznesof AW. Self-starvation of rats living in activity wheels on a restricted feeding schedule. J Comp Physiol Psychol 1967;64:414–21.
[41] Seeley RJ, Yagaloff KA, Fisher SL, Burn P, Thiele TE, van Dijk G, et al. Melanocortin receptors in leptin effects. Nature 1997;390:349.
[42] Small CJ, Kim MS, Stanley SA, Mitchell JR, Murphy K, Morgan DG, et al. Effects of chronic central nervous system administration of agouti-related protein in pair-fed animals. Diabetes 2001;50:248–54.
[43] Small CJ, Liu YL, Stanley SA, Connoley IP, Kennedy A, Stock MJ, et al. Chronic CNS administration of Agouti-related protein (Agrp) reduces energy expenditure. Int J Obes Relat Metab Disord 2003;27:530–3.
[44] Sullivan PF. Mortality in anorexia nervosa. Am J Psychiatry 1995;152:1073–4.
[45] Vaisse C, Clement K, Durand E, Hercberg S, Guy-Grand B, Froguel P. Melanocortin-4 receptor mutations are a frequent and heterogeneous cause of morbid obesity. J Clin Invest 2000;106:253–62.
[46] Vergoni AV, Bertolini A. Role of melanocortins in the central control of feeding. Eur J Pharmacol 2000;405:25–32.
[47] Vink T, Hinney A, van Elburg AA, van Goozen SH, Sandkuijl LA, Sinke RJ, et al. Association between an agouti-related protein gene polymorphism and anorexia nervosa. Mol Psychiatry 2001;6:325–8.
[48] Von Frijtag JC, Croiset G, Gispen WH, Adan RA, Wiegant VM. The role of central melanocortin receptors in the activation of the hypothalamus-pituitary-adrenal-axis and the induction of excessive grooming. Br J Pharmacol 1998;123:1503–8.
[49] Wong ML, Licinio J, Gold PW, Glowa J. Activity-induced anorexia in rats does not affect hypothalamic neuropeptide gene expression chronically. Int J Eat Disord 1993;13:399–405.