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AgRP$_{(83—132)}$ and SHU9119 differently affect activity-based anorexia

Jacquelien J.G. Hillebrand [a,b], Martien J.H. Kas [a], Anton J.W. Scheurink [c], Gertjan van Dijk [c], Roger A.H. Adan [a,*]

[a] *Department of Pharmacology and Anatomy, Rudolf Magnus Institute of Neuroscience, University Medical Center Utrecht, Universiteitsweg 100, 3584 CG Utrecht, The Netherlands*
[b] *Altrecht Rintveld Eating Disorders, Zeist, The Netherlands*
[c] *Department of Neuroendocrinology, University of Groningen, The Netherlands*

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Abstract Activity-based anorexia (ABA) mimics starvation and hyperactivity of anorexia nervosa patients in rats. Activation of the melanocortin (MC) system leads to hypophagia and increased energy expenditure in ad libitum fed rats. Therefore, activation of the MC system might underlie the development and propagation of ABA. Pro-opiomelanocortin (POMC) gene expression is normally decreased during negative energy balance. Strikingly, we found a transient up-regulation of POMC mRNA levels in the arcuate nucleus during the development of ABA, indicating a hyperactive MC system. However, wheel running and food intake were not influenced by treating ABA rats with the competitive antagonist SHU9119. This suggests that agonism of MC receptors by endogenous α-melanocyte-stimulating hormone (α-MSH) levels does not underlie ABA. Instead, treatment with the inverse agonist AgRP$_{(83—132)}$ did ameliorate signs of ABA. This implies that modulation of constitutive MC receptor activity rather than antagonizing putative α-MSH release contributes to the development and propagation of ABA.

1. Introduction

Anorexia nervosa (AN) is a psychiatric disorder and is often characterized by extreme hypophagia, obsessive fears of being fat and hyperactivity (Casper et al., 1991; Kron et al., 1978; Walsh and Devlin, 1998). The mechanisms underlying the development of AN are poorly understood. Still the severity of AN, being the psychiatric disorder with the highest mortality rate, asks for attention. Research on AN used to be focused on psychological characteristics; however, nowadays, more attention is given to a putative role of biological parameters (Frank et al., 2002; Hebebrand et al., 2003; Klein and Walsh, 2005).

* Corresponding author. Tel.: +31 302538517; fax: +31 302539032.
E-mail address: r.a.h.adan@med.uu.nl (R.A.H. Adan).

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The activity-based anorexia (ABA) paradigm serves as an animal model of AN. In the ABA model, rats have continuous access to running wheels but have limited access to food. Over the course of daily scheduled feeding, rats increase their running wheel activity (RWA). Enhanced RWA is specifically observed a few hours prior to food access, a phenomenon, which is described as food-anticipatory activity (FAA) (Mistlberger, 1994). Additionally, rats decrease food intake, lose body weight as well as body temperature and the hypothalamus–pituitary–adrenal (HPA) axis is activated (Burden et al., 1993; Hall and Hanford, 1954; Hillebrand et al., 2005a; Routtenberg and Kuznesof, 1967).

It is not known which mechanisms underlie the imbalance in energy intake and energy expenditure in ABA rats. The central melanocortin (MC) system is a possible candidate system involved in this paradoxical behavior. This system consists of pro-opiomelanocortin (POMC) neurons, agouti-related protein (AgRP) neurons and MC3 and MC4 receptors (MC-Rs) (Desarnaud et al., 1994; Gantz et al., 1993; Kishi et al., 2003; Mountjoy et al., 1992, 1994; Roselli-Rehfuss et al., 1993). MC peptides are encoded by the POMC transcript and act as agonists of the central MC3 and MC4-Rs, whereas endogenous AgRP is an inverse agonist (or antagonist) of these receptors (Haskell-Luevano and Monck, 2001; Nijenhuis et al., 2001). POMC cells are localized in the arcuate nucleus (ARC) of the hypothalamus and in the nucleus of the solitary tract of the brainstem (and peripherally in the pituitary) (Gee et al., 1983; Jacobowitz and O'Donohue, 1978). POMC neurons are inhibited in response to fasting (Bergendahl et al., 1992) and stimulated in the overfed state (Hagan et al., 1999). In contrast, AgRP neurons in the ARC are stimulated by fasting and inhibited by leptin treatment (Mizuno and Mobbs, 1999). Activation of the MC system by central injections of the endogenous agonist α-melanocyte-stimulating hormone (α-MSH) results in hypophagia, increased energy expenditure and activation of the HPA axis (Forbes et al., 2001; Haynes et al., 1999; Vergoni and Bertolini, 2000; Von Frijtag et al., 1998). Central injections of the synthetic competitive antagonist SHU9119 antagonize these effects (Adage et al., 2001; Fan et al., 1997). Thus, central activation of the MC system influences parameters (feeding, energy expenditure and HPA axis activation) that are also affected in ABA rats.

We recently showed that central infusion of α-MSH enhances development of ABA and that central infusion of $AgRP_{(83-132)}$ increases survival rate of ABA in rats (Hillebrand et al., 2005b; Kas et al., 2003). We also observed that NDP-MSH binding is increased in the ventromedial nucleus of the hypothalamus (VMH) upon 1 week of exposure to the ABA paradigm. This indicates increased MC binding sites in the VMH and thus increased activity of the MC system. A down-regulation of POMC gene expression following 1 week exposure to the ABA paradigm was observed (Kas et al., 2003), but information on temporal changes in the expression profile of this neuropeptide over the course of ABA development is lacking.

In AN patients, cerebrospinal fluid levels of POMC protein are decreased, but are normalized after recovery (Kaye et al., 1987). Recently, auto-antibodies against the MC system were detected in plasma of AN patients, which could possibly interfere with MC signaling and contribute to pathophysiology (Fetissov et al., 2002). Polymorphisms in the MC4-R gene (Val103Ile, Ile251Leu) and AgRP gene (Ala67Thr) have also been found in AN patients. Although the occurrence of the MC4-R polymorphism was not different from a control population (Hinney et al., 1999), the AgRP polymorphism occurred more frequently in a population of AN patients compared to controls (Vink et al., 2001) and was associated with low body weight in two other populations (Argyropoulos et al., 2002; Marks et al., 2004).

In the present study, we investigated the role of the MC system in development and propagation of ABA. First, we examined temporal changes in POMC gene expression during the development of ABA. We found an unexpected transient up-regulation of POMC gene expression in rats exposed to the ABA paradigm. Second and third, we examined the importance of this finding by treating ABA rats with the competitive antagonist SHU9119 and the inverse agonist $AgRP_{(83-132)}$.

2. Experimental procedures

2.1. Rats

Female outbred Wistar WU rats (Harlan, Horst, The Netherlands) were individually housed in a temperature and humidity controlled room (21 ± 2 °C) under a 12:12-h dark/light cycle (ZT12 = lights off). Food and water were ad libitum available unless stated otherwise. The ethical committee on use and care of animals of the University of Utrecht approved all described procedures. Rats eating less than 4 g chow on 2 consecutive days were removed from the experiment before day 6 for ethical reasons and were excluded from analysis.

2.2. Drugs

SHU9119 (0.5 μg/day, Bachem, Bubendorf, Switzerland, similar to Adage et al., 2001) (Experiment 2) and $AgRP_{(83-132)}$ (human, 5.6 μg/day icv, Phoenix Pharmaceuticals, Belmont, CA, USA, similar to Kas et al., 2003) (Experiment 3) were dissolved in sterile isotonic saline (=vehicle) and chronically infused (12 μl/day) into the lateral ventricle using osmotic minipumps (Alzet Model 1007D + brain infusion kit 3–5 mm, DURECT Corporation, Cupertino, CA, USA).

2.3. Surgical procedures

One week after arrival, rats (Experiments 2 and 3) received radio telemetric transmitters (TA10TA-F40 Data Sciences International, St. Paul, MN, USA) in the abdominal cavity under 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 ip) anaesthesia. All rats were treated with buprenorphin (Temgesic®, Schering-Plough, Maarssen, The Netherlands, 0.05 ml/100 g sc) and saline (1 ml sc) after surgery, and were allowed to recover for 2 weeks.

For intracerebroventricular (icv) surgery (Experiments 2 and 3), rats were anaesthetized using Hypnorm® (0.1 ml/100 g im). The head was shaved and the skull was exposed by a midline incision of the skin. After preparation of a small craniotomy (approximately 1 mm in diameter) by high-

speed drilling, a brain infusion cannula was placed into the lateral ventricle 1 mm lateral, 1 mm posterior from bregma and fixed to the skull with two small screws and dental cement. The cannula was connected by tubing (filled with vehicle or ligand) to an osmotic minipump containing vehicle or ligand. Minipumps were subcutaneously placed into the flank region after overnight incubation at 37 °C. After surgery, rats were treated with Temgesic® (0.02 ml/100 g sc) and saline (1 ml sc).

2.4. Experimental set-up

2.4.1. Experiment 1: POMC gene expression during development of ABA

After acclimatization (1 week) to the animal facility, rats ($n=29$) were individually housed in cages with running wheels for an adaptation period of 10 days (days −10 to −1) under ad libitum feeding conditions. Running wheel activity (RWA) was continuously recorded using a Cage Registration Program (Dept. Biomedical Engineering, UMC Utrecht, The Netherlands) (average RWA days −4 to −1: 7052.4 ± 525.4 revolutions/day, average body weight day −1: 215.6 ± 2.1 g). Food was removed at day 0 (ZT12) and was accessible again at day 1 (ZT12) during 1 h, whereas the water bottle and the running wheel were continuously accessible. Body weight (ZT11) and food intake (ZT13) were measured daily. This procedure lasted for 4 days. On each experimental day, selected rats ($n=4$–5 per day, matched for body weight (day −1) and baseline RWA (day −4 to day −1)) were decapitated at ZT11. Brains were rapidly removed, quickly frozen in cold (−35 °C) isopentane and stored at −80 °C. Cryosections (coronal, 20 μm) of the ARC were thaw-mounted onto RNAse free poly-L-lysine coated slides. The slides were stored at −80 °C until processing for in situ hybridization.

In an additional experiment, sedentary rats ($n=12$, bw day −1: 222.3 ± 5.2) were pair-fed to ABA rats for 3 days. Each day, four rats were sacrificed (matched for body weight day −1) as described above.

2.4.2. Experiment 2: SHU9119 treatment in ABA rats

After acclimatization (1 week) and recovery from telemetric surgery (see above, 2 weeks), rats ($n=16$) were individually housed in cages with running wheels under ad libitum feeding conditions for an adaptation period of 10 days (days −10 to −1). RWA was continuously recorded using the Cage Registration Program. On day −1, transmitters were switched on by magnetic field induction to allow continuous assessment of body temperature. Rats were divided into two groups (1 h food + vehicle or 1 h food + SHU9119), matched for 4-day RWA (average day −4 to day −1: 3792.7 ± 562.8 revolutions/day) and body weight (average day −1: 231.4 ± 3.8 g). Icv cannulae and osmotic minipumps were implanted as described above. After surgery (day 0, ZT12), food was removed from all cages. The next days, rats had 1-h access to food (ZT12 to ZT13), while water was continuously available. Body weight (ZT11) and food intake (ZT13) were measured daily. At the end of day 6 (ZT11), rats were decapitated. Trunk blood was collected in lithium-heparin containing tubes (Sarstedt, Nümbrecht, Germany) after adding 83 μmol EDTA and 1 mg aprotonin. Plasma was separated and frozen at −20 °C until further determination. Adrenal glands were isolated and weighed.

In an additional experiment, icv cannulae were implanted in ad libitum fed sedentary rats (average bw day −1: 204.1 ± 3.1 g) and were connected to osmotic minipumps containing vehicle or SHU9119 using the same procedures as described above. These rats ($n=10$) had ad libitum access to food following surgery. Body weight and food intake were measured daily (ZT11). Rats were sacrificed after 4 days of SHU9119 treatment as described above.

2.4.3. Experiment 3: $AgRP_{(83–132)}$ treatment in ABA rats

After acclimatization, recovery from telemetric surgery and adaptation, rats ($n=14$) were divided into two groups (1 h food + vehicle or 1 h food + $AgRP_{(83–132)}$), matched for 4-day RWA (average day −4 to day −1: 7542.3 ± 1107.1 revolutions/day) and body weight (day −1: 228.5 ± 3.3 g). Further procedures were similar as described in Experiment 2.

2.5. In situ hybridization

Cryostat brain sections of rats (Experiment 1) were all used in the same assay. 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. A ^{33}P-labeled RNA probe (^{33}P-UTP, ICN Diagnostics, Zoetermeer, The Netherlands) was made using a 350 bp rat POMC cDNA fragment (Kas et al., 2003). The sections were hybridized overnight at 72 °C with 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 ammonium acetate. Sections were exposed to X-ray film (Kodak Bio-Max MR) for 5 days. The films were developed and expression of POMC was semi-quantitatively analyzed using a calibration curve and the Microcomputer Imaging Device (MCID) (Imaging Research Inc., St. Catharines, Ontario, Canada).

2.6. Radioimmunoassay

To investigate activation of the HPA axis (Experiments 2 and 3), plasma levels of corticosterone and adrenocorticotropic hormone (ACTH) (day 6, ZT11) were analyzed by radioimmunoassay (RIA). A commercially rat RIA kit was used to measure plasma corticosterone levels (ICN Biochemicals, Costa Mesa, CA, USA). Plasma ACTH levels were 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, California, USA) was labeled with ^{125}I and used as a tracer (Nijsen et al., 2000).

2.7. Data analysis

All data are presented as mean ± standard error and were analyzed using the statistical programs R and SPSS 11.5.

Data were controlled for normality and homogeneity. For all measurements, baseline levels were not different between vehicle-treated and ligand-treated groups. In Experiment 1, changes in POMC gene expression were analyzed by ANOVA using least significant difference (LSD) as a post hoc test. Relative body weight (=body weight as % of day −1) and RWA during the light phase and during the dark phase were analyzed by repeated measurements in R.

One rat (Experiment 2: SHU9119) and two rats (Experiment 3: vehicle, $AgRP_{(83-132)}$), respectively, were sacrificed before day 6 due to ethical reasons and were excluded from analysis. To record basal resting body temperature levels, body temperature was measured by telemetry and was analyzed as average body temperature during at least 30 min of inactivity in the light phase (ZT0–ZT3). Relative body weight (=body weight as % of day −1), food intake, RWA and body temperature were analyzed by repeated measures analysis using Huynh Feldt correction for Mauchlys sphericity effects, followed by *t*-tests. Cumulative food intake, final body weight, plasma ACTH, plasma corticosterone and adrenal weights were analyzed by *t*-tests. Differences were considered significant at $P<0.05$.

3. Results

3.1. POMC gene expression during development of ABA

We measured POMC gene expression in the ARC during initial days of exposure to the ABA paradigm. Strikingly, POMC expression was transiently up-regulated in the ARC during development of ABA [$F(5,22)=3.95$, $P=0.02$] (Fig. 1A). POMC mRNA levels were increased at days 0, 1 and 2 ($P<0.01$) as compared to baseline (=day −1). On days 3 and 4, POMC gene expression levels decreased, but were not different from baseline.

During development of ABA, relative body weight decreased to 73.1 ± 0.8% on day 4 (days 0–4: $P<0.01$) (Fig. 1B). RWA increased during the dark phase (days 1–4: $P=0.01$) and light phase (days 2–4: $P<0.01$) as compared to day −1 (=baseline) (Fig. 1C+D).

To verify that early up-regulation of POMC was specific for ABA rats, we pair-fed sedentary rats to ABA rats and measured POMC gene expression levels during 3 days. Pair-fed rats showed body weight loss (day 1: 94.8 ± 0.4%) (days 0 and 1: $P<0.01$), but no early up-regulation of POMC [$F(2,9)=0.19$, n.s.] as compared to baseline.

3.2. SHU9119 treatment in ABA rats

We measured the effect of chronic treatment with the competitive MC antagonist SHU9119 on development and propagation of ABA. Statistical analysis revealed no effects of SHU9119 treatment over time (days 1–6) on food intake [$F(5,65)=0.62$, n.s.] and relative body weight (days 0–6) [$F(6,78)=0.21$, n.s.] of ABA rats. Cumulative food intake (days 1–6) [$t(13)=-1.22$, n.s.] and final relative body weight [$t(13)=0.66$, n.s.] were also not different between SHU9119-treated and vehicle-treated ABA rats (Fig. 2A–C). Basal body temperature decreased following scheduled feeding, but did not differ between SHU9119-treated and vehicle-treated rats over time [$F(6,78)=1.36$, n.s.] (Fig. 2D).

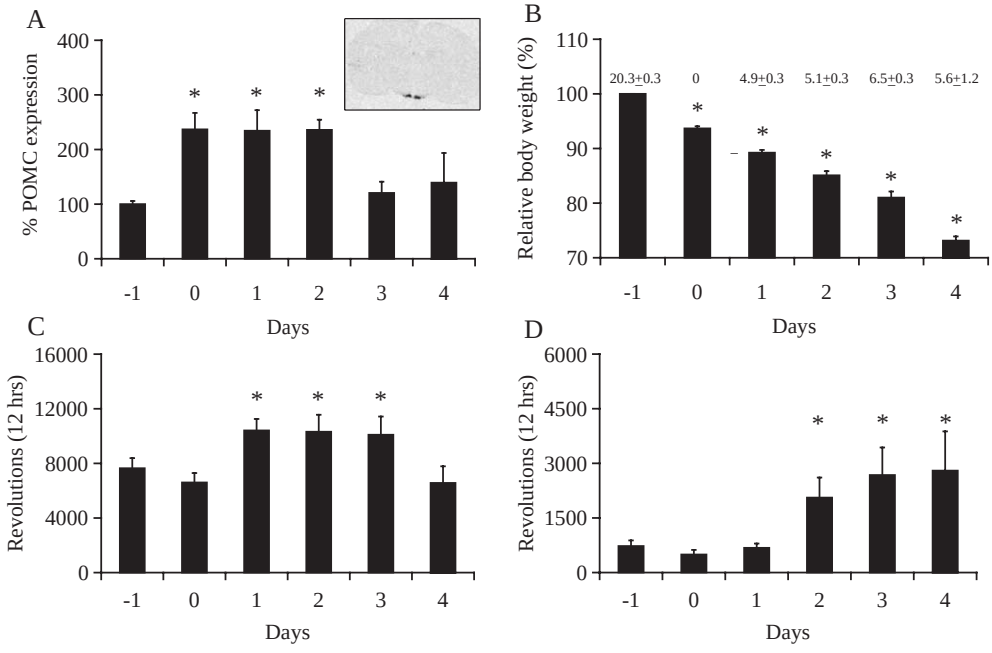

Figure 1 POMC gene expression, relative body weight, food intake and running wheel activity during the development of activity-based anorexia (ABA). (A) Pro-opiomelanocortin (POMC) mRNA levels in the arcuate nucleus ($n=4$–5 per day) were measured during development of ABA by radioactive in situ hybridization and expressed as % of day −1. ANOVA, LSD. (B) Relative body weight (% of day −1) was measured at ZT11. The daily food intake is depicted above each bar. (C) Running wheel activity during the dark phase and (D) during the light phase were analyzed during development of ABA ($n=4$–5 per day). *T*-test, $P<0.05$. *Different from day −1 (=ad libitum fed running). Values are means ± S.E.

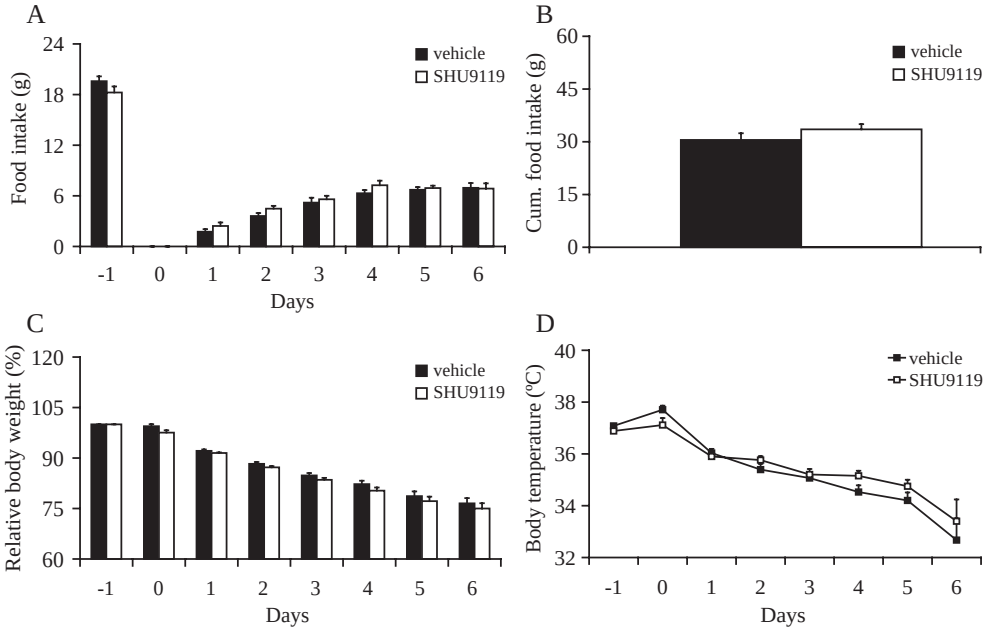

Figure 2 Food intake, relative body weight and basal body temperature in vehicle-treated and SHU9119-treated activity-based anorexia rats (ABA). (A) Daily food intake, (B) cumulative food intake (days 1—6), (C) relative body weight and (D) basal body temperature in vehicle-treated ($n=8$) and SHU9119-treated (0.5 μg/day) ($n=7$) rats exposed to the ABA model. Food restriction and infusion started on day 0. Basal body temperature was measured during at least 30 min of inactivity in the early light phase. *T*-test, $P<0.05$. Values are means ± S.E.

Daily RWA [$F(6,78)=0.59$, n.s.], dark phase RWA [$F(6,78)=0.59$, n.s.] and light phase RWA [$F(6,78)=0.89$, n.s.] were not influenced by SHU9119 treatment over time. Cumulative daily RWA (days 0—6) [$t(13)=-0.56$, n.s.], dark RWA [$t(13)=-0.17$, n.s.] and light RWA [$t(13)=-0.96$, n.s.] were also not influenced by SHU9119 treatment (Fig. 3A—D).

Moreover, plasma ACTH levels [$t(10)=0.24$, n.s.], plasma corticosterone levels [$t(13)=0.69$, n.s.] and relative adrenal

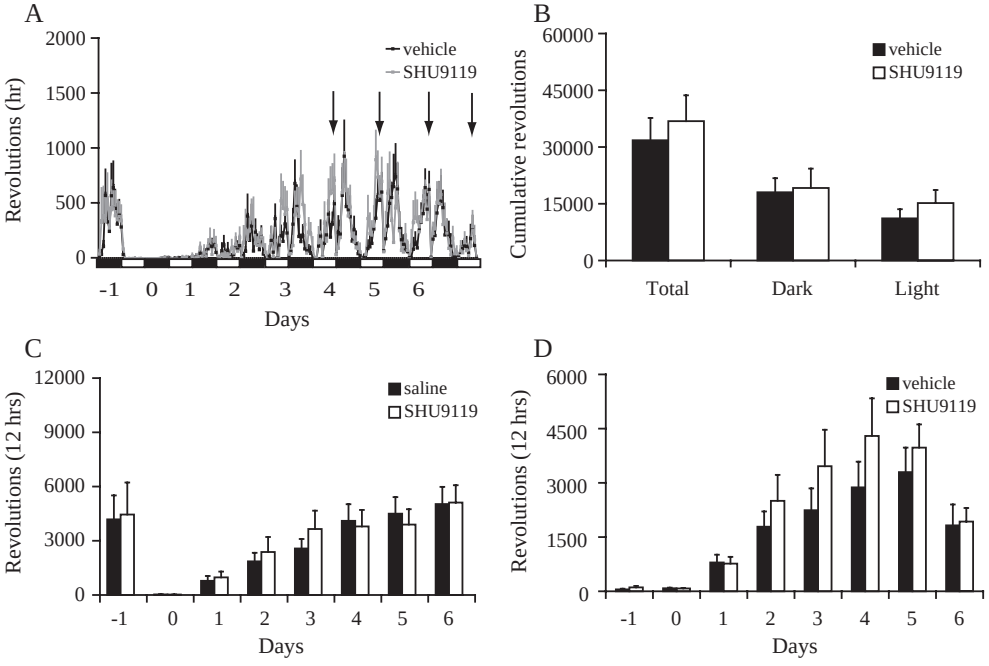

Figure 3 Running wheel activity (RWA) in vehicle-treated and SHU9119-treated activity-based anorexia rats (ABA). (A) Total revolutions per hour; (B) cumulative revolutions (days 0—6) during the whole day, light phase and dark phase; (C) revolutions during the dark phase; and (D) revolutions during the light phase following vehicle ($n=8$) or SHU9119 (0.5 μg/day) ($n=7$) infusion. Food restriction and infusion started on day 0. Food-anticipatory activity (FAA) is indicated by arrows. *T*-test, $P<0.05$. Values are means ± S.E.

Table 1 HPA axis characteristics of vehicle-treated and SHU9119-treated ABA rats

Treatment	ACTH (pg/ml)	Corticosterone (μg/dl)	Adrenal weight (%)
Vehicle ($n=8$)	176.7 ± 32.7	35.1 ± 2.6	0.042 ± 0.003
SHU9119 ($n=7$)	163.9 ± 9.2	32.2 ± 3.2	0.038 ± 0.002

Plasma adrenocorticotropic hormone (ACTH, day 6), plasma corticosterone levels (day 6) and relative adrenal weight (% of body weight, day 6) in vehicle-treated and SHU9119-treated (0.5 μg/day) rats exposed to the activity-based anorexia model. *T*-test, $P<0.05$. Values are means ± S.E.

gland weight [$t(12)=0.83$, n.s.] in ABA rats were not changed by 1 week SHU9119 treatment as compared to vehicle treatment (Table 1).

To ascertain the efficacy of SHU9119 to induce hyperphagia, ad libitum fed sedentary rats were chronically treated with the same dose of SHU9119. SHU9119-treated rats showed increased food intake and body weight gain (4-day food intake: 109.5 ± 10.6 g, 4-day body weight gain: 38.2 ± 6.3 g) as compared to vehicle-treated rats (4-day food intake: 72.8 ± 4.3 g, 4-day body weight gain: 10.6 ± 1.4 g) (food $t(8)=-3.22$, $P=0.01$; body weight gain $t(8)=-2.73$, $P=0.03$).

3.3. $AgRP_{(83-132)}$ treatment in ABA rats

We measured the effect of chronic treatment with the inverse agonist $AgRP_{(83-132)}$ on development and propagation of ABA. There were no significant effects of treatment over time on food intake (days 1–6) [$F(5,50)=1.40$, n.s.] and relative body weight (days 0–6) [$F(6,60)=1.04$, n.s.] in ABA rats. However, cumulative food intake (days 1–6) [$t(10)=-4.73$, $P<0.01$] was increased in $AgRP_{(83-132)}$-treated rats, whereas final relative body weight was not different [$t(10)=-0.89$, n.s.] (Fig. 4A–C). Basal body temperature was affected by $AgRP_{(83-132)}$ treatment over time [$F(6,60)=3.37$, $P=0.03$]. $AgRP_{(83-132)}$-treated rats had a higher basal body temperature than vehicle-treated rats during ABA (Fig. 4D).

Daily RWA [$F(6,60)=1.18$, n.s.], dark phase RWA [$F(6,60)=0.85$, n.s.] and light phase RWA [$F(6,60)=1.31$, n.s.] were not affected by $AgRP_{(83-132)}$ treatment over time. Also cumulative daily RWA (days 0–6) [$t(10)=-0.49$, n.s.], light RWA [$t(10)=-1.30$, n.s.] and dark RWA [$t(10)=-0.12$, n.s.] were not affected (Fig. 5A–D).

One week of $AgRP_{(83-132)}$ treatment did not influence HPA axis activation. Plasma ACTH levels [$t(10)=-1.05$, n.s.], plasma corticosterone levels [$t(10)=0.91$, n.s.] and relative adrenal glands weight [$t(9)=0.01$, n.s.] were not affected by $AgRP_{(83-132)}$ treatment in ABA rats (Table 2).

4. Discussion

This study determined changes in POMC gene expression in ABA rats as well as the effects of SHU9119 and $AgRP_{(83-132)}$ treatment on behavioral and physiological parameters in ABA rats. Strikingly, a transient increase in POMC mRNA expression was observed in the ARC of rats exposed to the ABA paradigm, which was not observed in pair-fed rats without a running wheel. After the third day of food restriction, POMC expression decreased. In line with previous work, a further

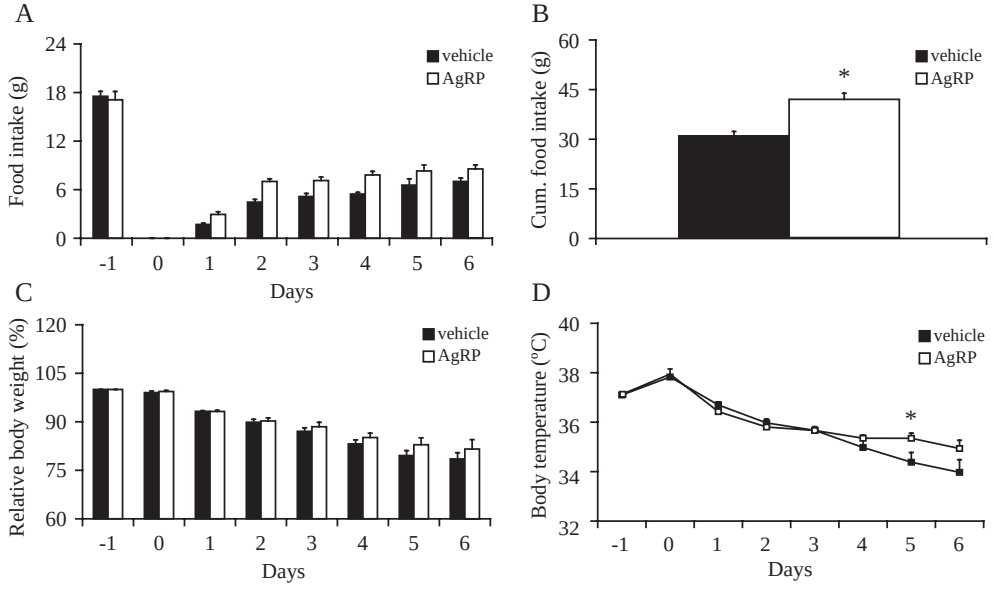

Figure 4 Food intake, relative body weight and basal body temperature in vehicle-treated and $AgRP_{(83-132)}$-treated activity-based anorexia rats (ABA). (A) Daily food intake, (B) cumulative food intake (days 1–6), (C) relative body weight and (D) basal body temperature in vehicle-treated ($n=6$) and $AgRP_{(83-132)}$-treated (5.6 μg/day) ($n=6$) rats exposed to the ABA model. Food restriction and infusion started on day 0. Basal body temperature was measured during at least 30 min of inactivity in the early light phase. *T*-test, $P<0.05$. Values are means ± S.E.

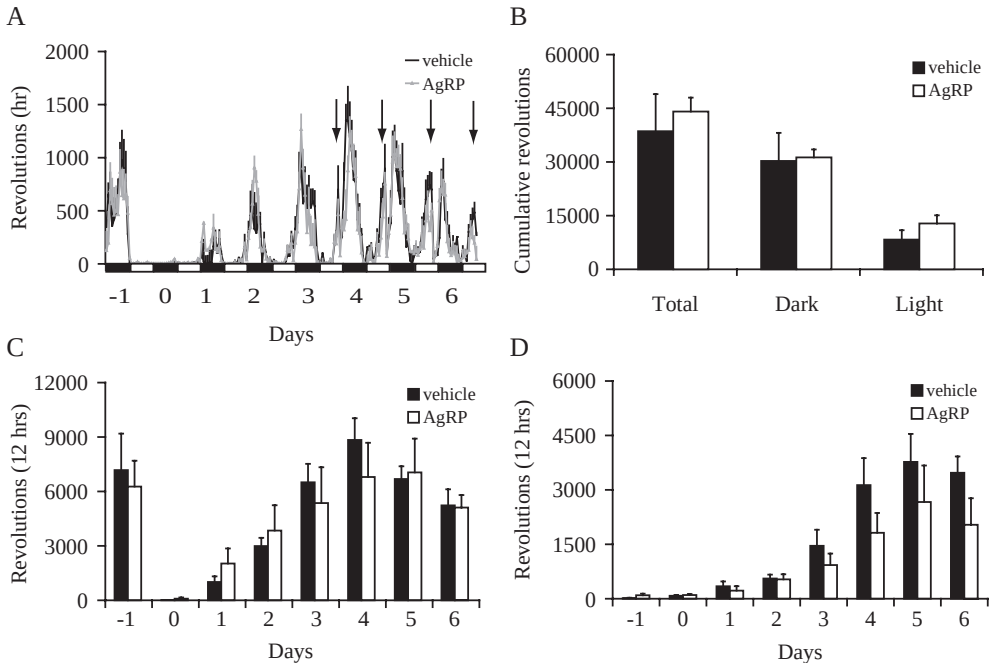

Figure 5 Running wheel activity (RWA) in vehicle-treated and $AgRP_{(83–132)}$-treated activity-based anorexia rats (ABA). (A) Total revolutions per hour; (B) cumulative revolutions (days 0–6) during the whole day, light phase and dark phase; (C) revolutions during the dark phase; and (D) revolutions during the light phase following vehicle ($n=6$) or $AgRP_{(83–132)}$ (5.6 μg/day) ($n=6$) infusion. Food restriction and infusion started on day 0. Food-anticipatory activity (FAA) is indicated by arrows. *T*-test, $P<0.05$. Values are means ± S.E.

decrease of POMC mRNA levels is expected when the experiment would have continued a few more days (Kas et al., 2003).

Up-regulation of POMC mRNA contrasts with the widely described down-regulation of anorexigenic neuropeptides during food restriction (Schwartz et al., 1997) and is probably a result of a unique situation of limited food access along with physical activity. Previously, we observed that physical activity per se does not result in up-regulation of POMC gene expression (Hillebrand et al., 2005c; Kas et al., 2003). One may argue that circadian fluctuation of POMC mRNA expression can also play a role in up-regulation of POMC. Previous work indicated that POMC mRNA levels in the early light phase are lower (Xu et al., 1999) than prior to the dark phase, when we measured POMC gene expression. However, this daily pattern of POMC gene expression is abolished during chronic scheduled feeding (Xu et al., 1999).

The mechanisms underlying transient POMC up-regulation remain unclear. Since up-regulation of POMC takes place when body weight decreases and RWA increases, changes in POMC gene expression might contribute to the typical paradoxical behavior in ABA, i.e. increased RWA and decreased food intake. Transient up-regulation of POMC mRNA and the increase of MC binding sites in the VMH (Kas et al., 2003) indicate a maladaptation of the MC system during ABA. These data suggest that the MC system remains active despite body weight loss and that the expected down-regulation of the MC system immediately following food restriction to conserve energy does not occur.

Transient up-regulation of POMC gene expression during development of ABA could be associated with increased release of α-MSH, which might contribute to the development of hyperactivity and anorexia. Recently, we showed that chronic infusion of exogenous α-MSH results in an enhancement of the development of ABA by reducing food intake and increasing FAA (Hillebrand et al., 2005b). To test the hypothesis that increased endogenous α-MSH release during the first days of exposure to the ABA model contributed to the development of ABA, rats were treated with the competitive MC-R antagonist SHU9119. However, food intake and RWA were not influenced by SHU9119 treatment, neither were body weight loss and body temperature. This was an unexpected finding since (the same dose of) SHU9119 induced a strong hyperphagic response in ad libitum fed rats.

Table 2 HPA axis characteristics of vehicle-treated and $AgRP_{(83–132)}$-treated ABA rats

Treatment	ACTH (pg/ml)	Corticosterone (μg/dl)	Adrenal weight (%)
Vehicle ($n=6$)	152.5 ± 26.5	33.5 ± 1.9	0.039 ± 0.002
$AgRP_{(83–132)}$ ($n=6$)	166.7 ± 21.3	32.8 ± 7.7	0.039 ± 0.003

Plasma adrenocorticotropic hormone (ACTH, day 6), plasma corticosterone levels (day 6) and relative adrenal weight (% of body weight, day 6) in vehicle-treated and $AgRP_{(83–132)}$-treated (5.6 μg/day) rats exposed to the activity-based anorexia (ABA) model. *T*-test, $P<0.05$. Values are means ± S.E.

Chronic infusions of $AgRP_{(83–132)}$ did not influence RWA or body weight loss in ABA rats. However, $AgRP_{(83–132)}$ strongly increased 1-h food intake and reduced starvation-induced hypothermia. Previously, we reported that chronic infusion of (the same dose of) $AgRP_{(83–132)}$, starting 60 h following surgery, decreased self-starvation in ABA (Kas et al., 2003). Kas et al. showed that $AgRP_{(83–132)}$-treated ABA rats had a higher body temperature as compared to vehicle-treated controls, which preceded the increase in food intake. In the present experiment, $AgRP_{(83–132)}$ was infused immediately following surgery and the effects of $AgRP_{(83–132)}$ on food intake and body temperature were confirmed. However, increased food intake was observed prior to changes in body temperature, suggesting that the decline in hypothermia is a result of increased food intake. Alternatively, the two studies suggest that $AgRP_{(83–132)}$ independently influences food intake and body temperature in ABA rats. Recently, it was shown that body temperature is an important parameter in ABA; influencing body temperature by changing ambient/cage temperature influences the development of ABA and has strong implications for survival (Gutierrez et al., 2002; Hillebrand et al., 2005a). In the present study, we found changes in body temperature following $AgRP_{(83–132)}$ treatment, but no effect on survival rate of ABA rats as we reported before (Kas et al., 2003). This might be explained by the use of rats with a higher body weight at baseline in the present experiment for ethical reasons; namely survival of rats in the ABA model is positively correlated with initial body weight.

The difference in effects of SHU9119 and $AgRP_{(83–132)}$ on development of ABA is remarkable. Both SHU9119 and $AgRP_{(83–132)}$ are often described as MC antagonists; however, their effects on central MC-Rs are different. Whereas SHU9119 acts as a competitive antagonist, with modest partial agonism at the MC3-R (Hruby et al., 1995), $AgRP_{(83–132)}$ acts as an inverse agonist, decreasing constitutive activity of MC3-R and MC4-R (Adan and Kas, 2003; Haskell-Luevano and Monck, 2001; Nijenhuis et al., 2001). Both compounds induce hyperphagia in ad libitum fed rats (Adage et al., 2001; Small et al., 2001). However, $AgRP_{(83–132)}$ but not SHU9119 increased feeding and body temperature in ABA rats. Besides, while both compounds did not significantly affect RWA, they tended to influence RWA oppositely. The data suggest that suppressing constitutive MC-R activity (by $AgRP_{(83–132)}$) rather than antagonizing α-MSH (by SHU9119) contributes to ABA. There are, however, several limitations to this study. We only used a single dose of SHU9119 and $AgRP_{(83–132)}$ (although we ascertained hyperphagic properties of this dose). It can also not be excluded that differences in pharmacokinetic properties or differences in other pharmacodynamic properties between $AgRP_{(83–132)}$ and SHU9119 at central MC-Rs contribute to the observed effects.

The long-lasting hyperphagic response of $AgRP_{(83–132)}$ is observed in ad libitum fed rats as well as food-restricted rats (Rossi et al., 1998; Wirth and Giraudo, 2000), however, the long-lasting hyperphagic response of SHU9119 (Fan et al., 1997) is absent in food-restricted or food-deprived rats (Williams et al., 2004). This suggests the existence of different mechanisms (e.g. additional to MC-Rs) mediating $AgRP_{(83–132)}$-induced and SHU9119-induced feeding. Requisitory actions of μ- and κ-opioid receptors necessary for $AgRP_{(83–132)}$ effects might be such a mechanism (Brugman et al., 2002; Hagan et al., 2001).

Based upon the transient increase in POMC mRNA levels and enhancement of ABA following exogenous administration of α-MSH (Hillebrand et al., 2005b), it was hypothesized that endogenous increases in α-MSH release would be involved in development of ABA. Although actual endogenous levels of α-MSH during ABA were not measured, the absence of effects of SHU9119 treatment indicates rejection of the hypothesis, keeping in mind the limitations of the study. The exact role of the transient up-regulation of POMC is thus not yet clarified, neither is the enhancement of ABA by exogenous α-MSH. These data urge for further investigations on the involvement of other POMC-derived neuropeptides in the development of ABA, like β-endorphin. β-Endorphin stimulates food intake as well as rewarding processes and nociception (Amalric et al., 1987; Glass et al., 1999; Kalra and Horvath, 1998; Mucha and Herz, 1985; Przewlocki and Przewlocka, 2001). Yet a possible anorexic potency of β-endorphin was discovered by generating (male) β-endorphin-deficient mice, which are obese and hyperphagic (Appleyard et al., 2003). Plasma and arcuate levels of β-endorphin are increased in ABA rats (Aravich et al., 1993). Additionally, it was recently discovered that μ-opioid receptor-deficient mice (which have reduced β-endorphin signaling) show reduced anticipatory activity during scheduled food access (Kas et al., 2004). Hence, the endogenous opioid system might be involved in the effects of $AgRP_{(83–132)}$ and the transient increase in POMC gene expression. Future studies will further address the role of β-endorphin and opioid signaling in ABA. Furthermore, other pathways involved in energy balance will be studied, which might interfere with the transient up-regulation of POMC and/or influence the development and/or propagation of ABA on their own as well.

In summary, POMC mRNA levels were up-regulated following early food restriction in ABA rats. This suggests activation of the MC system, which seems counterintuitive since MC activation results in hypophagia and increased energy expenditure. Chronic SHU9119 treatment did not influence ABA, suggesting that putative increased agonism of MC-Rs by endogenous α-MSH does not play a major role in ABA. Instead, chronic $AgRP_{(83–132)}$ treatment increased food intake and body temperature in the ABA model. Hence, suppression of constitutive receptor activity by $AgRP_{(83–132)}$ may explain the observed effects. Interestingly, it was recently shown that the N-terminal domain of the human MC4-R acts as a partial agonist resulting in constitutive MC4-R signaling (Srinivasan et al., 2004), which further supports the relevance of constitutive MC4-R signaling in vivo.

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