Hypothalamic neuropeptide expression following chronic food restriction in sedentary and wheel-running rats

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Abstract

When rats are given access to a running-wheel in combination with food restriction, they will become hyperactive and decrease their food intake, a paradoxical phenomenon known as activity-based anorexia (ABA). Little is known about the regulation of the hypothalamic neuropeptides that are involved in the regulation of food intake and energy balance during the development of ABA. Therefore, rats were killed during the development of ABA, before they entered a state of severe starvation. Neuropeptide mRNA expression levels were analysed using quantitative real-time PCR on punches of separate hypothalamic nuclei. As is expected in a state of negative energy balance, expression levels of agouti-related protein (AgRP) and neuropeptide Y (NPY) were increased 5-fold in the arcuate nucleus (ARC) of food-restricted running ABA rats vs 2-fold in sedentary food-restricted controls. The co-regulated expression of AgRP and NPY strongly correlated with relative body weight and white adipose tissue mass. Arcuate expression of pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) was reduced 2-fold in the ABA group. In second-order neurons of the lateral hypothalamic area (LHA), melanin-concentrating hormone (MCH) mRNA expression was upregulated 2-fold in food-restricted running rats, but not in food-restricted sedentary controls. Prepro-orexin, CART and corticotropin-releasing hormone expression levels in the LHA and the paraventricular nucleus (PVN) were unchanged in both food-restricted groups. From this study it was concluded that during the development of ABA, neuropeptides in first-order neurons in the ARC and MCH in the LHA are regulated in an adequate response to negative energy balance, whereas expression levels of the other studied neuropeptides in secondary neurons of the LHA and PVN are unchanged and are probably regulated by factors other than energy status alone.

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Introduction

The hypothalamus has a major role in the regulation of food intake and energy balance. Several hypothalamic nuclei, including the arcuate nucleus (ARC), paraventricular nucleus (PVN), lateral hypothalamic area (LHA), ventromedial nucleus (VMH) and dorsomedial nucleus (DMH), express neuropeptides that are involved in the regulation of food intake and energy balance. ARC neurons are first-order neurons in the hypothalamic response to the peripheral satiety factors leptin and insulin. First-order ARC neurons project to second-order neurons in the PVN, LHA, VMH and DMH. Second-order neurons subsequently project to various regions including the nucleus of the solitary tract and the dorsomotor nucleus of the vagus in the caudal brainstem as well as to the cortex and the limbic system. The caudal brainstem harbours the basic neural circuitry required for eating reflexes, but needs hypothalamic input for the long-term regulation of energy homeostasis (Schwartz *et al.* 2000, Hillebrand *et al.* 2002, Berthoud 2004).

There are at least two different populations of first-order neurons in the ARC. One population contains the orexigenic neuropeptides agouti-related protein (AgRP) and neuropeptide Y (NPY). Expression of AgRP and NPY is suppressed in states of positive energy balance when leptin and insulin levels are high.

In contrast, leptin and insulin stimulate the expression of pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART), which are co-expressed in the second group of first-order neurons in the ARC. POMC is the precursor of the melanocortin peptide α -melanocyte-stimulating hormone (α -MSH), which, like CART, has an inhibitory effect on food intake (reviewed in Hillebrand *et al.* 2002, Leibowitz & Wortley 2004).

AgRP and NPY mRNA expression is increased during 1–7 days of fasting and after food restriction in mice (Hahn *et al.* 1998, Mizuno *et al.* 1998, Mizuno & Mobbs 1999, Morton *et al.* 2004) and rats (Brady *et al.* 1990, Korner *et al.* 2001, Bertile *et al.* 2003, Bi *et al.* 2003, Wolden-Hanson *et al.* 2004). Both AgRP and NPY are upregulated in the leptin-deficient *ob/ob* mouse

(Shutter *et al.* 1997, Mizuno *et al.* 1998, Mizuno & Mobbs 1999, Yamamoto *et al.* 1999, Tritos *et al.* 2001). The effects of fasting on arcuate POMC and CART expression are less pronounced. POMC and CART expression is unchanged or decreased after 1–7 days of fasting in rats (Brady *et al.* 1990, Kristensen *et al.* 1998, Kaneda *et al.* 2001, Korner *et al.* 2001, Li *et al.* 2002, Savontaus *et al.* 2002, Bertile *et al.* 2003, Bi *et al.* 2003, Wolden-Hanson *et al.* 2004). In *ob/ob* mice, POMC and CART levels are decreased relative to wild-type mice (Mizuno *et al.* 1998). Also nociceptin, a neuropeptide with orexigenic properties, is expressed in the ARC (reviewed in Olszewski & Levine 2004). However, nociceptin expression is not increased after a 16 h fast (Rodi *et al.* 2002). Taken together, first-order neurons are strongly regulated by disturbances in energy balance, which is most explicit in the increased AgRP and NPY expression during fasting and the increased POMC and CART expression by leptin.

Neuropeptides expressed in second-order neurons include CART (PVN, LHA & DMH), melanin-concentrating hormone (MCH; co-expressed with CART in the LHA), orexins (LHA), corticotropin-releasing hormone (CRH; PVN) and nociceptin (PVN, LHA, VMH and DMH). MCH, orexins and nociceptin stimulate food intake (although less when compared with NPY), whereas CRH is an anorexigenic neuropeptide (Hillebrand *et al.* 2002, Leibowitz & Wortley 2004, Olszewski & Levine 2004).

The regulation of neuropeptide expression in second-order neurons by disturbances in energy balance is less prominent than in first-order neurons. Fasting results in elevated or unchanged expression of MCH and orexin in the LHA (Presse *et al.* 1996, Qu *et al.* 1996, Herve & Fellmann 1997, Sakurai *et al.* 1998, Cai *et al.* 1999, Lopez *et al.* 2000, Swart *et al.* 2001, Tritos *et al.* 2001, Bertile *et al.* 2003, Akiyama *et al.* 2004, Morton *et al.* 2004). In *ob/ob* mice MCH expression is upregulated, whereas orexin expression has been reported to be either downregulated or unaffected (Qu *et al.* 1996, Yamamoto *et al.* 1999, 2000, Tritos *et al.* 2001). As in the ARC, nociceptin expression in the PVN and the LHA was unchanged after a 16 h fast (Rodi *et al.* 2002). Expression of anorexigenic CRH in the PVN was either decreased or unchanged following fasting or food restriction (Brady *et al.* 1990, Kiss *et al.* 1994, Ahima *et al.* 1999, Fekete *et al.* 2000, Kaneda *et al.* 2001). Also CART expression in the PVN is decreased after fasting, whereas CART expression in the LHA remains unaffected (Li *et al.* 2002, Wolden-Hanson *et al.* 2004). The discrepancies in the nature and extent of regulation of neuropeptide expression following fasting suggest that factors other than energy balance *per se* influence gene regulation of these neuropeptides. These discrepancies may be explained by differences in the animals like strain, sex or age, and differences in experimental conditions, which

besides the length of the fasting period may include single or group housing, circadian time of killing animals and environmental conditions such as the presence of a running-wheel.

Activity-based anorexia (ABA) is an animal model that is used to study aspects of anorexia nervosa. In this model, rats are given free access to a running-wheel and are at the same time exposed to a restricted feeding schedule. As a consequence, rats become hyperactive and decrease their food intake (Routtenberg & Kuznesof 1967). Depending on, amongst others, the severity of the feeding schedule, rats lose more than 25% of their body weight within a week upon exposure to the ABA model (Burden *et al.* 1993, Kas *et al.* 2003) and eventually will not survive (Routtenberg & Kuznesof 1967, Kas *et al.* 2003). A hallmark of ABA is not only the development of hyperactivity, but also the shift in activity patterns. ABA rats display substantial activity during the period preceding scheduled feeding. This food-anticipatory activity (FAA) seems crucial for the development of ABA (Dwyer & Boakes 1997). ABA also results in activation of the hypothalamo–pituitary–adrenal (HPA) axis, which manifests itself in increased adrenal weight and plasma corticosterone levels (Burden *et al.* 1993). As yet there is no conclusive theory explaining the mechanism behind the paradox of self-starvation and hyperactivity leading to physical collapse in ABA.

Little is known about the regulation of the hypothalamic neuropeptides that are involved in the regulation of feeding behaviour and energy balance (e.g. candidate neuropeptides) during ABA. After a week of exposure to the ABA model, POMC expression in the ARC is decreased whereas arcuate AgRP expression is strongly upregulated (Kas *et al.* 2003). CRH expression in the PVN is unchanged when rats are exposed to ABA until they reach a relative body weight of 75% (Burden *et al.* 1993). The problem is that in both of those studies rats had already developed severe ABA. The aim of the present study was to investigate the regulation of candidate hypothalamic neuropeptides in first- and second-order neurons during the earlier stages of the development of ABA and to compare these results with those of sedentary food-restricted rats. To this extent, rats were killed at the end of the fourth day of exposure to the ABA model, before they entered a state of severe emaciation. Neuropeptide mRNA expression levels were then measured using quantitative real-time PCR (qPCR) on punches containing separate hypothalamic regions.

Materials and methods

Animal experiments

Female outbred Wistar WU rats ($n=32$, synchronised for oestrous cycle; Harlan, Horst, The Netherlands) weighing approximately 160 g upon arrival were

Figure 1 Schedule of the experiment. Filled bars indicate the darkness phase, open bars the light phase.

individually housed under a 12 h light:12 h darkness cycle in a room with constant ambient temperature ($21 \pm 2^\circ\text{C}$). Rats were allowed to habituate to this environment for 2 weeks, during which food and water were available freely. After these 2 weeks, 16 rats were placed in cages containing a running-wheel for a 10 day adaptation period while the 16 other rats were left in their home cages. Food and water remained available freely. After this 10 day adaptation period, both running and sedentary rats were divided into two groups matched for 4 day running-wheel activity (RWA; average days $-4/-1$: 5730 ± 753 revolutions) and body weight (average day -1 : 215 ± 1.6 g). For both running and sedentary rats, one group retained free access to food and water while the other group was exposed to a feeding schedule in which food was available for 1 h at the onset of the dark phase only. Figure 1 shows a schedule of the experiment. Body weight was measured just before the onset of the dark phase, and just before food access in the food-restricted groups. RWA was continuously monitored using a Cage Registration program (Department of Biomedical Engineering, UMC Utrecht, The Netherlands). Rats were sacrificed by decapitation at the end of the light phase of day 3. Trunk blood was collected into tubes containing lithium-heparin (Sarstedt, Nümbrecht, Germany) with $83 \mu\text{mol}$ EDTA and 1 mg aprotinin. Tubes were collected on ice until centrifugation (20 min at 2600 g at 4°C); subsequently plasma was stored at -20°C until further processing. Brains were quickly removed, frozen in cold isopentane and stored at -80°C . Retroperitoneal white adipose tissue (WAT) and WAT surrounding the oviducts, interscapular brown adipose tissue (BAT) and adrenals were collected and weighed.

All described procedures were approved by the ethical committee on use and care of animals of the University of Utrecht, The Netherlands.

RIAs

Plasma leptin and insulin were measured using commercially available rat RIA kits, according to the manufacturer's protocol (Linco Research, St Charles, MO, USA).

Microdissection

Coronal sections of $300 \mu\text{m}$ thickness were cut from frozen rat brains in a cryostat at approximately -8°C , transferred to a flexible black rubber strip and immediately covered with RNAlater reagent (Ambion Europe, Huntingdon, UK). Punches containing the selected hypothalamic regions were taken with a stainless steel punch needle of 2 mm diameter (Zivic Laboratories, Pittsburgh, PA, USA). For each region, punches were pooled for each individual rat. Specific sections from which punches were taken based on the rat brain atlas coordinates of Paxinos & Watson (1998). PVN punches ($n=4$ per rat) were taken from two adjacent sections between -1.7 and -2.4 mm relative to bregma. ARC punches ($n=3$ per rat) were taken from three subsequent sections between -2.6 and -3.8 mm relative to bregma. LHA punches ($n=4$ per rat) were taken from two adjacent sections between -3.0 and -3.8 mm relative to bregma, from the same sections as from which ARC punches were taken. Punches were stored at 4°C in RNAlater for a maximum of 48 h until further processing.

RNA isolation and cDNA synthesis

RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The quality of the RNA was determined by agarose gel electrophoresis. RNA quality of samples was regarded acceptable for further use if the 18S and 28S ribosomal RNAs appeared intact. cDNA was synthesised from total RNA that had been treated with RNase free DNaseI (Roche, Mannheim, Germany) using SuperScript II reverse transcriptase and oligo dT₁₂₋₁₈ primers (Invitrogen). No dithiothreitol was used in the reaction as it may negatively influence the efficiency of the subsequent PCRs (Lekanne Deprez *et al.* 2002).

qPCR

qPCR was performed using a LightCycler instrument and the LightCycler FastStart DNA Master SYBR Green I reaction mix (Roche), according to the manufacturer's instructions. Reactions were performed in a total volume of $10 \mu\text{l}$ containing $2 \mu\text{l}$ cDNA,

Table 1 Overview of qPCR primers (f: forward primer; r: reverse primer), gene accession numbers of sequences used as template or references from which primer sequences were taken, product lengths, annealing temperatures, optimal MgCl₂ concentrations and qPCR efficiencies (E)

Gene	Gene accession no./reference	Primers 5'–3'	Product (bp)	Annealing Temperature (°C)	MgCl ₂ (mM)	E
AgRP	AF206017	f: AGCAGACCGAGCAGAAGATG r: GACTCGTGCAAGCTTACACA	114	61	5	1.95
NPY	NM012614	f: TATCCCTGCTCGTGTGTTTG r: GTTCTGGGGGCATTTTCTG	220	57	3	1.96
POMC	AF510391	f: ACCTCACACGGAAGCAA r: CGGGGATTTTCAGTCAAGG	118	54	4	1.94
CART	Marie-Claire <i>et al.</i> (2003)	f: GCCAAGTCCCCATGTGTGAC r: CACCCCTTCACAAGCACTTCA	129	60	4	1.95
Nociceptin	U48262	f: TGGTAATGTGTAGCCAGAAGGA r: AGGATGAGCAAGAAGATGGG	259	64	3	2.03
CRH	Wei & Sternberg (2004)	f: TGGATCTCACCTTCCACCTTCTG r: CCGATAATCTCCATCAGTTTCCTG	103	62	4	1.89
MCH	M29712	f: TCGGTTGTTGCTCCTTCTCT r: TTCCCTCTTTTCTGTGTGG	225	57	3	1.98
Prepro-orexin	Johren <i>et al.</i> (2002)	f: GCCGTCTCTACGAAGTGTG r: CGAGGAGAGGGGAAGTTAG	303	56	3	1.86

2–5 mM MgCl₂, 0.5 μM of each primer and 1 μl of the SYBR Green reaction mix. Primer sequences were taken from the literature or designed with Primer3 software using settings recommended in the LightCycler manual. For each primer pair, annealing temperature and MgCl₂ concentration were optimised. PCR efficiencies were obtained from standard curves of serial cDNA dilutions over three to four orders of magnitude. Primer sequences, annealing temperatures, MgCl₂ concentrations and PCR efficiencies are listed in Table 1. Specificity of the PCRs were verified using melting curve analysis and agarose gel electrophoresis, confirming the formation of a single PCR product of the expected length. cDNA samples from separate nuclei from single rats were measured in duplicate. Cycle threshold values were obtained using the second derivative maximum method in the LightCycler software. Gene expression was calculated as normalised ratio (R_n), which is a measure of the amount of cDNA present in a sample relative to a calibrator sample measured in the same qPCR experiment and normalised to a reference gene. For each nucleus studied, a pool from cDNAs from that nucleus was used as calibrator sample. Cyclophilin was used as reference gene (Medhurst *et al.* 2000).

Data analysis

All data are presented as means ± S.E.M. Statistical analysis was performed using SPSS 11.5 software. Total RWA, food intake, food intake on day 3, WAT and BAT weights and relative adrenal weights were analysed by ANOVA using Bonferroni's post-hoc test. Relative body weight was analysed by GLM repeated-measures

analysis followed by ANOVA and Bonferroni's post-hoc test. Except for nociceptin, neuropeptide mRNA expression levels between freely fed and food-restricted rats were analysed using a one-sided *t*-test as expression of these neuropeptides has been shown to be consistently either up- or downregulated upon fasting or food restriction. Nociceptin levels were compared using a two-sided *t*-test. Plasma leptin and insulin levels between freely fed vs food-restricted rats, as well as between freely fed sedentary vs running rats, were analysed using a one-sided *t*-test. Differences were considered significant at $P<0.05$. Correlations between physiological parameters and neuropeptide expression levels were determined using Pearson's bivariate correlation analysis.

Results

ABA parameters

Rats with access to running-wheels developed anticipatory activity before the scheduled hour of feeding (Fig. 2), while total RWA over the food restriction period was not significantly increased ($P=0.163$, *t*-test) in food-restricted rats relative to freely fed running rats (Table 2). Over the 4 days during which rats were subjected to the model, as well as in the last feeding period before the experiment was stopped, rats in running-wheels did not eat less than food-restricted sedentary rats (Table 2). The relative body weights of food-restricted rats decreased over time. This effect was stronger in running rats than in sedentary rats (Fig. 3). Available WAT and BAT stores decreased following food restriction. WAT stores were also decreased in

Figure 2 Hourly RWA in food-restricted rats ($n=8$) and freely fed rats ($n=8$). Black bars at the x-axis indicate the darkness phase, white bars the light phase.

freely fed running rats as compared with freely fed sedentary rats. Relative adrenal weight was increased following food restriction in running rats. Relative adrenal weight was also increased in freely fed running rats as compared with freely fed sedentary rats (Table 2).

Neuropeptide expression in hypothalamic nuclei

As shown in Fig. 4, AgRP and NPY mRNA expression levels in the ARC were significantly increased in both sedentary and running food-restricted rats as compared

with freely fed rats. This effect was stronger in the running animals, in which AgRP and NPY were increased 4.8- and 5.4-fold respectively vs 2.3- and 1.7-fold in the sedentary groups.

Expression levels of other neuropeptides in ARC, PVN and LHA, expressed as R_n , are plotted in Fig. 5 for all experimental groups. The anorexigenic neuropeptides in the ARC, POMC and CART were both significantly downregulated in restricted running rats (2.1- and 1.5-fold respectively), but only CART was significantly downregulated in restricted sedentary rats

Table 2 Physiological parameters and RWA. Starting body weight (day -1), total RWA, total food intake, food intake on day 3, WAT and BAT weights and relative adrenal weights in sedentary and running freely fed and food-restricted rats ($n=8$ per group) are listed.

	Sedentary freely fed	Sedentary food-restricted	Running freely fed	Running food-restricted
Day -1 body weight (g)	216±3.3	216±3.9	213±3.8	216±2.3
Total RWA (revolutions)			29566±6254	40492±3997
Cumulative food intake (g)	80.3±1.9 ^a	13.3±1.0 ^b	87±3.0 ^a	16.7±0.90 ^b
Day 3 food intake (g)	19.2±0.58 ^a	5.4±0.52 ^b	21.8±0.85 ^c	5.9±0.40 ^b
WAT (mg)	5810±540 ^a	3368±522 ^{bc}	3922±302 ^b	1669±467 ^c
BAT (mg)	299±20 ^a	179±16 ^b	249±15 ^a	134±6 ^b
Adrenal weight (% body weight day 3)	0.027±0.001 ^a	0.033±0.002 ^{ab}	0.032±0.001 ^b	0.041±0.002 ^c

Different superscript letters indicate significant differences (one-way ANOVA, followed by Bonferroni's post-hoc test, differences were considered significant at $P<0.05$)

Figure 3 Relative body weights (means $\pm$ S.E.M.) of food-restricted and freely fed sedentary rats and food-restricted and freely fed running rats (all $n=8$). Different letters indicate significant differences over time (GLM repeated-measures analysis followed by ANOVA and Bonferroni's post-hoc test with differences considered significant at $P<0.05$).

(1.6-fold) as compared with freely fed rats. Expression levels of nociceptin in the ARC, as well as expression levels of CART, CRH and nociceptin in the PVN and of prepro-orexin and CART in the LHA were not

significantly different in both sedentary and running food-restricted animals as compared with freely fed rats. A small but significant upregulation of MCH (1.3-fold) was found in the LHA of food-restricted running rats but not in food-restricted sedentary rats. Both plasma leptin and insulin levels were reduced in food-restricted groups. In freely fed running rats, plasma leptin and insulin levels were lower than in freely fed sedentary rats (Fig. 4).

Correlation analysis

The variations in gene expression levels of AgRP and NPY were especially large in the group of food-restricted running rats (Fig. 4). In order to investigate to what extent variability in AgRP and NPY mRNA expression were explained by differences in body weight loss between rats, correlation analysis was performed within this group. AgRP and NPY gene expression levels showed a strong negative correlation with relative body weight and WAT mass (Table 3), but not with insulin, whereas correlation analysis with plasma leptin levels did not reach significance ($P=0.051$ for AgRP and $P=0.054$ for NPY). Moreover, expression levels of AgRP and NPY were strongly correlated ($r=0.995$, $P<0.001$, $n=8$)

Figure 4 (A–E) R_n of AgRP and NPY in the ARC in freely fed (ad lib) and food-restricted (restr) sedentary (sed) and in freely fed and food-restricted running (run) rats; (A, B) show data for individual rats, (D, E) show group means $\pm$ S.E.M. (C, F) Mean $\pm$ S.E.M. group levels of plasma insulin and leptin levels respectively. * $P<0.05$, ** $P<0.01$, one-sided t -test.

Figure 5 Neuropeptide mRNA expression levels (means $\pm$ S.E.M.) in ARC, PVN and LHA of sedentary and running freely fed and food-restricted rats expressed as R_n ($n=4-8$ per group; significant differences between freely fed and food-restricted rats, * $P<0.05$, ** $P<0.01$, one-sided t -test).

within the group of food-restricted running rats, as well as in the other groups (data not shown).

Correlation analysis was also performed on POMC and CART expression as these neuropeptides, like AgRP and NPY, are co-localised in the ARC. Despite considerable variability in POMC as well as CART expression levels, no correlation was found between POMC and CART expression levels in the ARC in any of the groups (data not shown). Plasma leptin levels were positively correlated with CART mRNA but not with POMC mRNA in the ARC when data from all groups were pooled together. Further correlation analysis within the group of food-restricted running rats showed strong negative correlations between arcuate nociceptin expression and relative body weight ($r = -0.927$, $P = 0.001$,

$n=8$) and WAT mass ($r = -0.828$, $P = 0.011$, $n=8$) and between POMC expression in the ARC and CRH expression in the PVN ($r = -0.938$, $P = 0.006$, $n=6$).

Expression levels of MCH and CART, which are co-localised in second-order neurons in the LHA, were significantly correlated in all groups except the food-restricted running rats (Table 4).

For none of the peptides studied did expression levels correlate with total RWA, RWA on day 3 or FAA on day 3 (data not shown).

Discussion

Food-restricted rats with access to a running-wheel developed characteristics of ABA. They exhibited

Table 3 Results of correlation analysis between arcuate AgRP and NPY expression and relative body weights (% day -1) and WAT mass (mg) on day 3 in food-restricted running rats

	Factor	<i>r</i>	<i>P</i>
Gene			
AgRP	relative BW	-0.900	0.002
NPY	relative BW	-0.891	0.003
AgRP	WAT	-0.861	0.006
NPY	WAT	-0.822	0.012

($n=8$; r : Pearson correlation coefficient; P : significance of the correlation, two-sided)

Table 4 Results of correlation analysis between expression levels of MCH and CART in the LHA

	<i>r</i>	<i>P</i>	<i>n</i>
Group			
Sedentary freely fed	0.795	0.033	7
Sedentary restricted	0.898	0.002	8
Running freely fed	0.942	0.000	8
Running restricted	0.611	0.108	8

(r : Pearson correlation coefficient; P : significance of the correlation, two-sided)

anticipatory activity before the scheduled hour of feeding, which is one of the major hallmarks of ABA (Dwyer & Boakes 1997). Furthermore, there was a strong decrease in relative body weight, available fat stores and plasma leptin and insulin levels. Relative adrenal weight was increased, indicating increased HPA axis activity, another characteristic of ABA (Burden *et al.* 1993). Rats were sacrificed after 4 days of food restriction, when they had not yet become anorexic as compared with sedentary controls and total RWA was not significantly increased relative to freely fed controls, despite the development of anticipatory activity. Consequently, neuropeptide gene expression was studied during a phase in which rats were still developing ABA. Changes found in the expression levels of hypothalamic neuropeptide genes should thus be indicative of the development of ABA and not of general exhaustion. Rats were sacrificed just before the scheduled feeding period. Therefore, peptide expression was expected to reflect food intake in the ensuing feeding period, which in previous experiments was the first time-point at which food-restricted rats in running-wheels ate less than sedentary food-restricted rats (J J G Hillebrand, unpublished observations).

Body composition was different between freely fed sedentary rats and freely fed running rats, the latter having less WAT, but equal body weight (Table 2, Fig. 3). Therefore, the effects of food restriction were compared within the running and sedentary groups separately.

In both running and sedentary food-restricted rats, AgRP and NPY mRNAs were increased. The upregulation was stronger in the running rats, reflecting a stronger negative energy balance in these animals. Correlation analysis within the group of running food-restricted rats showed that both AgRP and NPY expression levels are negatively correlated with relative body weight and WAT. This indicates that AgRP and NPY expression is dependent on the availability of energy stores. The fact that we found no correlation between AgRP or NPY mRNA levels and plasma insulin and leptin levels within this group of rats, may have been caused by the fact that all rats within this group had similarly low levels of these hormones. The negative correlation between the expression of AgRP and NPY and WAT correlates with a strong reduction in the level of circulating leptin, which is produced by adipose tissue (reviewed in Mohamed-Ali *et al.* 1998) and is reduced in ABA (Hillebrand *et al.* 2005). There was also a strong correlation between AgRP and NPY expression levels in all groups. Taken together, it can be concluded that the expression of AgRP and NPY is co-regulated and increases during the development of ABA, which is an adequate response to negative energy balance. Interestingly, AgRP and NPY expression levels in the ARC did not differ between running and sedentary freely fed rats,

despite significant differences in plasma leptin and insulin levels between these groups. This indicates that factors other than leptin and insulin regulate expression of these mRNAs.

Expression of the anorexigenic neuropeptides POMC and CART was downregulated in the ARC of food-restricted running rats. Thus, as for AgRP and NPY, expression of POMC and CART in the ARC appears to be regulated in an adequate manner during the development of ABA. However, although POMC and CART are co-localised in the same arcuate neurons, only CART was significantly downregulated in the ARC of food-restricted sedentary rats and expression levels of POMC and CART did not correlate in any of the groups. This suggests that regulation of expression between POMC and CART is more differentiated than regulation of AgRP and NPY, which are strongly co-regulated by leptin and energy balance. Interestingly, plasma leptin levels were positively correlated with CART mRNA but not with POMC mRNA in the ARC when data from all groups were pooled together. Thus, other factors besides energy balance and leptin may regulate POMC expression in the ARC. One such factor may be the serotonin system, since POMC/CART neurons are activated by stimulation of the serotonin 5HT_{2C} receptors that are expressed on the majority of these neurons (Heisler *et al.* 2002).

In the present study, expression of nociceptin in the ARC was not changed during chronic food restriction. This is in line with the effect of short term (16 h) food restriction, which does not lead to changed nociceptin expression either (Rodi *et al.* 2002). However, correlation analysis showed that arcuate nociceptin expression was negatively correlated with relative body weight and WAT mass in food-restricted running rats. This may indicate that arcuate nociceptin expression is upregulated during strong negative energy balance, although this effect is not as strong as the regulation of AgRP and NPY.

MCH in the second-order neurons in the LHA was selectively upregulated during food restriction in running rats but not in sedentary rats, indicating that MCH expression is increased in situations of severe negative energy balance. This has also been shown by Bertile *et al.* (2003) who found that MCH expression during fasting was not increased until after more than 4 days of fasting. Nociceptin expression was modestly increased in the LHA of food-restricted running rats, but expression of the other neuropeptides studied in the LHA, CART and prepro-orexin, was not changed during food restriction in either sedentary or running rats. This is in accord with data obtained after 48 and 16 h of fasting for CART and nociceptin respectively (Li *et al.* 2002, Rodi *et al.* 2002) and with reports on prepro-orexin regulation during food restriction (Cai *et al.* 1999, Akiyama *et al.* 2004). Thus, in contrast to first-order neurons in the

ARC, in second-order neurons in the LHA, only MCH expressions and nociceptin are regulated by strong negative energy balance. Strong negative energy balance also dissociates the regulation of MCH and CART in the LHA. Therefore, factors other than energy status may play a more important role in regulating neuropeptides in second-order neurons in the LHA. For instance, MCH expression is inhibited by oestrogen (Murray *et al.* 2000, Mystkowski *et al.* 2000).

Food restriction did not result in changes in expression levels of CART, nociceptin and CRH in the PVN. For CART, this is in contrast with the effects of 48–72 h fasting, which decreases CART expression in the PVN (Li *et al.* 2002, Wolden-Hanson *et al.* 2004). For nociceptin this corresponds with the effect of 16 h of fasting (Rodi *et al.* 2002). Unchanged expression levels of CRH in the PVN in ABA have been reported before (Burden *et al.* 1993, Wong *et al.* 1993). Interestingly, CRH expression in the PVN was negatively correlated with POMC expression in the ARC in the food-restricted running animals. This is unexpected regarding previous findings. α -MSH prevents fasting-induced suppression of CRH expression in the PVN (Fekete *et al.* 2000) and the melanocortin agonist MTII induces c-Fos expression in the PVN (Thiele *et al.* 1998). These findings suggest a positive correlation between POMC and CRH expression. During development of ABA, the control of CRH activation through POMC may thus be disrupted.

None of the neuropeptides that were investigated in first- and second-order neurons correlated with RWA or FAA. Since the atypical antipsychotic olanzapine, which amongst others acts on 5HT_{2A/C} receptors, strongly reduces RWA in ABA rats (Hillebrand *et al.* 2005) it is likely that other systems such as the serotonin system are involved in the development of hyperactivity seen in ABA.

In conclusion, during the development of ABA, the expression of orexigenic and anorexigenic neuropeptides in primary and secondary neurons in the ARC, PVN and LHA are regulated in a way that is expected in response to negative energy balance. Kas *et al.* (2003) have described that the expression of melanocortin receptors in the ventromedial hypothalamus is increased during ABA. Therefore, this study does not rule out involvement of the neuropeptides that were investigated here *per se* in the development of ABA, since it is possible that changes in neuropeptide receptor density rather than neuropeptide expression itself contribute to the development of ABA.

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