The appetite suppressant d-fenfluramine reduces water intake, but not food intake, in activity-based anorexia

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Abstract

Biochemical, genetic and imaging studies support the involvement of the serotonin (5-HT) system in anorexia nervosa. Activity-based anorexia (ABA) is considered an animal model of anorexia nervosa, and combines scheduled feeding with voluntary running wheel activity (RWA). We investigated the effect of d-fenfluramine (d-FEN) treatment on development and propagation of ABA. d-FEN is an appetite suppressant and acts on 5-HT_{2C} receptors that are located on pro-opiomelanocortin (POMC) neurons in the arcuate nucleus of the hypothalamus. Since stimulation activation of the melanocortin system stimulates ABA, we hypothesized that d-FEN treatment enhances the development and propagation of ABA. Rats were exposed to the ABA model and chronically infused with d-FEN. Unexpectedly, d-FEN-treated ABA rats did not reduce food intake or increase wheel running as compared with vehicle-treated ABA rats. Furthermore d-FEN treatment did not affect body weight loss, hypothalamus-pituitary-adrenal axis activation, or starvation-induced hyperthermia in ABA rats. POMC mRNA levels in d-FEN-treated rats were not different from vehicle-treated rats after one week of exposure to the ABA paradigm. However, d-FEN-treated ABA rats showed hypodipsia and increased plasma osmolality and arginine-vasopressin expression levels in the hypothalamus. We conclude that d-FEN treatment does not enhance ABA under the experimental conditions of this study, but strongly reduces water intake in ABA rats.

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Introduction

Anorexia nervosa (AN) patients often show extreme dieting, body weight loss and hyperactivity (Kron *et al.* 1978, Casper *et al.* 1991, Walsh & Devlin 1998). The serotonin (5-HT) system is involved in regulation of appetite and mood, and is the major biological system of interest in research on AN. For example, cerebrospinal fluid (csf) levels of 5-HT metabolite, 5-hydroxyindoleacetic acid (5-HIAA) are reduced in AN patients, while csf 5-HIAA levels are increased in recovered patients (Kaye *et al.* 1984, 1991). AN patients do not benefit from treatment with selective 5-HT reuptake inhibitors (SSRIs), but SSRI treatment appears to prevent relapse after weight restoration (Kaye *et al.* 2001). It was hypothesized by Kaye and colleagues that AN patients reduce food intake (thereby reducing dietary intake of the 5-HT precursor tryptophan) to diminish symptoms of their intrinsic dysphoric mood. Indeed, it was recently reported that plasma tryptophan levels are low in AN patients but increase following weight gain (Attia *et al.* 2005). Moreover, acute tryptophan depletion reduced anxiety levels in AN patients (Kaye *et al.* 2003). Association studies revealed

polymorphisms in the promoter of the 5-HT_{2A} and 5-HT_{2C} receptor that were associated with AN (Collier *et al.* 1997, Gorwood *et al.* 2002, Hu *et al.* 2003, Sorbi *et al.* 1998, Westberg *et al.* 2002), although this could not be confirmed by others (Campbell *et al.* 1998, Nacmias *et al.* 1999). In addition, results from positron emission tomography (PET) studies showed that AN patients have reduced 5-HT_{2A} receptor binding in the mesial temporal lobe, which persists after recovery (Frank *et al.* 2002).

The activity-based anorexia (ABA) model is considered an animal model of AN. In this model, food access restricted to one hour per day in combination with voluntary access to running wheels is reported to result in hyperactivity, hypophagia and severe body weight loss (Hall & Hanford 1954, Routtenberg & Kuznesof 1967). In addition to hyperactivity, rats show an altered activity pattern and develop extensive activity prior to food access, which is referred to as food-anticipatory activity (FAA) (Mistlberger 1994). Furthermore, it was shown before that ABA rats are hypothermic (Hillebrand *et al.* 2005a) and have an increased activation of the hypothalamus-pituitary-adrenal (HPA) axis (Burden *et al.* 1993, Kas *et al.* 2003). In an alike semi-starvation-induced hyperactivity (SIH) model (which uses 60% of

baseline food intake/day instead of 1 h food access/day), 5-HT turnover was increased in the medial basal hypothalamus (Broocks *et al.* 1991).

The melanocortin (MC) system plays an important role in energy homeostasis (Cone 1999, Schwartz *et al.* 2000). Expression levels of pro-opiomelanocortin (POMC) were decreased in the arcuate nucleus (ARC) of the hypothalamus of rats that were exposed to the ABA paradigm for one week (Kas *et al.* 2003). Recently, we observed that stimulation of the MC system by chronic treatment with the endogenous agonist α -melanocyte stimulating hormone (α -MSH, a neuropeptide derived from POMC), decreased food intake and increased FAA, body weight loss and activation of the HPA axis in ABA rats (Hillebrand *et al.* 2005b). Previously, we also showed that AgRP_(83–132) treatment prevented self-starvation in ABA rats, by influencing food intake, locomotor activity and body temperature (Kas *et al.* 2003).

D-fenfluramine (d-FEN) stimulates 5-HT release and blocks its reuptake (Rowland & Carlton 1986, Gibson *et al.* 1993, McCann *et al.* 1997). The 5-HT_{1B} and 5-HT_{2C} receptor are the main candidate receptors mediating the inhibitory control of 5-HT on food intake. Both these receptors also seem to be involved in d-FEN-induced hypophagia (Dourish 1995, Tecott *et al.* 1995, Simansky 1996, Clifton *et al.* 2000, Vickers *et al.* 2003). 5-HT_{1B} as well as 5-HT_{2C} deficient mice showed an attenuated response to d-FEN-induced hypophagia, although they still had d-FEN-induced locomotor deficits at high doses (Lucas *et al.* 1998, Vickers *et al.* 1999, Lee *et al.* 2004). Hypophagic actions of d-FEN were reduced by co-treatment with a 5-HT_{1B} or 5-HT_{2C} receptor antagonist (Vickers *et al.* 2001, Simansky & Nicklous 2002). Yet other studies showed that mainly the 5-HT_{2C} receptor plays an important role in d-FEN-induced hypophagia (Vickers *et al.* 2001, Clifton *et al.* 2003). It was recently demonstrated that 5-HT_{2C} receptors are located on POMC neurons in the ARC. Activation of POMC neurons by d-FEN resulted in an increased firing rate of these neurons and subsequently in an anorexic response (Heisler *et al.* 2002).

Given that interference with the MC system influences the development and propagation of ABA (Kas *et al.* 2003, Hillebrand *et al.* 2005b) and since d-FEN acts upstream of the MC system (Heisler *et al.* 2002), we hypothesized that d-FEN treatment enhances ABA by reducing food intake and increasing RWA, specifically FAA. Although d-FEN-induced hypophagia in ABA rats was reported before (Rieg *et al.* 1994), effects of d-FEN treatment on running wheel activity (RWA) have not been observed. In the present study we investigated the effect of d-FEN treatment on development and propagation of ABA further, by analyzing several characteristics of ABA such as ingestive behaviour (food and water intake), RWA (including FAA), body

temperature and activation of the HPA axis as well as hypothalamic POMC gene expression.

Materials and methods

Rats

Female outbred Wistar WU rats ($n=44$, Harlan, Horst, The Netherlands) were individually housed in a temperature and humidity controlled room ($21 \pm 2^\circ\text{C}$) 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.

Drugs

For *in vivo* administration, d-FEN (Servier, Paris, France) was dissolved in sterile isotonic saline and was chronically (continuous during 1 week) infused subcutaneously (sc) at a dose of 10 mg/kg/day using osmotic minipumps (0.5 $\mu\text{l/h}$, Alzet, model 1007D, DURECT Corporation, Cupertino, CA, USA). No adverse reactions to chronic administration of this dose of dFEN were reported before (Reig *et al.* 1994).

Surgical procedures

One week after arrival, rats received 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 intramuscular) and midazolam (Dormicum®, Hoffman-LaRoche, Mijdrecht, The Netherlands, 0.05 ml/100 g intraperitoneal) anaesthesia (ABA experiment only). After surgery, rats were treated with buprenorphin (Temgesic®, Schering-Plough, Maarsse, The Netherlands, 0.05 ml/100 g sc) and saline (1 ml sc).

For chronic vehicle or d-FEN infusions, all rats were anaesthetized by fentanyl/fluanisone as described above and the right flank was shaved. An osmotic minipump containing vehicle or d-FEN (10 mg/kg/day) was sc. placed into the flank region of the rat after overnight incubation at 37°C .

Experimental set-up

d-Fenfluramine treatment in ad libitum fed rats

In this experiment, the effects of chronic d-FEN treatment in *ad libitum* fed sedentary rats were investigated. Two weeks after arrival (day -1), 14 rats were divided into two groups (vehicle and d-FEN), matched for body weight (average body weight day -1 : 238.2 ± 2.5 g). Osmotic minipumps were implanted as described before. After surgery (day 0, ZT 12), food and

water were *ad libitum* available. Each day, body weight, food intake and water intake were measured at the end of the light phase (ZT11). Rats were decapitated after 1 week (day 6, ZT11). Trunk blood was collected into lithium-heparin containing tubes (Sarstedt, Nümbrecht, Germany) after adding 83 µmol EDTA and 1 mg aprotinin. 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.

d-Fenfluramine treatment in activity-based anorexia rats

In this experiment, the effects of chronic d-FEN treatment on development and propagation of ABA were investigated. After 2 weeks of recovery from the implantation of transmitters, 16 rats were placed into cages with running wheels for a training period of 10 days (day -10 to day -1) with *ad libitum* food and water access. RWA was continuously registered using a Cage Registration Program (Department of Biomedical Engineering, UMC Utrecht, The Netherlands). On day -1 , rats were divided into two groups (vehicle and d-FEN), matched for body weight (average body weight day -1 : 227.7 ± 2.7 g) and baseline RWA (average day-4 : day-1: 4024.5 ± 510.9 revolutions/day). Transmitters were switched on (by magnetic field induction) to allow continuous assessment of body temperature and locomotor activity (LMA). Osmotic minipumps were implanted as described before. After surgery (day 0, ZT12), food was removed, while water remained *ad libitum* available. Food was available during the first hour of the dark phase (ZT12 to ZT13) (day 1–6). Each day, body weight, food intake and water intake were measured. Rats were decapitated after 1 week (day 6, ZT11) as described above.

In situ hybridization

Cryosections (coronal, $20\text{ }\mu\text{m}$) of the hypothalamus of all rats were sliced using a cryostat (Leica, Rijswijk, The Netherlands) and thaw-mounted onto RNase free Superfrost slides (Menzel, Brannschweig, Germany). The slides were stored at -80°C until processed for *in situ* hybridization. Sections were fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 10 min, washed in PBS, pre-treated 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 -labelled antisense RNA probes were made using a 350 bp rat POMC cDNA fragment and a 205 bp arginine-vasopressin (AVP) cDNA fragment (exon C) (Kas *et al.* 2003). The sections were hybridized overnight at 72°C with 1×10^6 c.p.m. probe in buffer containing 50% deionized formamide, $2 \times$ standard saline citrate (SSC),

10% dextrane sulphate, $1 \times$ 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 $5 \times$ SSC (short, 72°C) and $0.2 \times$ 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, Amersham) for 5 days. The films were developed and expression of POMC (in the ARC) and AVP (in the paraventricular nucleus (PVN) and supraoptic nucleus (SON)) was semi-quantitatively analyzed using a calibration curve and the Microcomputer Imaging Device (MCID) (Imaging Research Inc., St Catharines, Ontario, Canada).

Radioimmunoassay

Plasma levels of corticosterone, adrenocorticotrophic hormone (ACTH) and leptin were analyzed by radioimmunoassay (RIA). A commercially available rat corticosterone RIA kit (ICN Biochemicals, Costa Mesa, CA, USA) and rat leptin RIA kit (Linco Research, St Charles, MO, USA, detection limit 0.5 ng/ml) were used. Assays were performed according to the manufacturer's protocol. Plasma ACTH was measured using a specific rabbit antiserum directed to the midportion of ACTH, which was kindly provided by Dr G B Makara (Budapest, Hungary). Synthetic human ACTH_(1–39) (Peninsula Laboratories, Belmont, CA, USA) was labelled with ^{125}I and used as a tracer (Nijssen *et al.* 2000).

Plasma osmolality

Plasma osmolality was measured by U-Diagnostics (Utrecht, The Netherlands) using a Fiske® 2400 multi-sample osmometer according to the manufacturer's protocol (Indumed, Dendermonde, Belgium).

Data analysis

All data are presented as mean $\pm$ S.E. Data were analyzed using SPSS 11.5 (SPSS Inc., Amsterdam, The Netherlands) for Windows and were controlled for normality and homogeneity. For all measurements, baseline levels were not significantly different between groups. Basal body temperature was analyzed as average body temperature (of telemetric recordings) during 30 min of inactivity in the early light phase (ZT0–ZT3). Relative body weight, food intake, water intake, RWA (ABA only), LMA (ABA only) and basal body temperature (ABA only) were analyzed by general linear model (GLM) repeated measures using Huynh-Feldt correction for Mauchly's sphericity effects and followed by *t*-tests. Cumulative RWA and LMA (ABA only), cumulative water and food intake, HPA axis activation, plasma osmolality and ISH data were analyzed by

t-tests. Differences were considered significant at $P < 0.05$.

Results

d-Fenfluramine treatment in *ad libitum* fed sedentary rats

Ad libitum fed sedentary rats were treated with 10 mg/kg/day d-FEN by osmotic minipumps for one week. This treatment significantly influenced food intake (day $F(6,72)=85.95$; $P < 0.001$, day $\times$ treatment $F(6,72)=16.06$; $P < 0.001$). d-FEN-treated rats had a reduced cumulative (day 0–6) food intake ($t(12)=7.05$; $P < 0.001$) as compared with vehicle-treated rats (Figure 1A).

Relative body weight was affected by chronic d-FEN treatment (day $F(6,72)=14.40$; $P < 0.001$, day $\times$ treatment $F(6,72)=2.52$; $P = 0.03$). d-FEN-treated rats had a lower relative body weight following 1 week as compared with vehicle-treated rats ($t(12)=4.16$; $P = 0.001$) (Figure 1B). Body weight loss was also evident from reduced plasma leptin levels following d-FEN treatment ($t(11)=2.97$; $P = 0.01$) (Table 1).

Water intake was also influenced by d-FEN treatment (day $F(6,66)=9.96$; $P < 0.001$, day $\times$ treatment $F(6,66)=14.41$; $P < 0.001$). Cumulative (day 0–6) water intake ($t(11)=3.56$; $P = 0.004$) was significantly reduced in d-FEN-treated rats ($73.2 \pm 4.5\%$) as compared with vehicle-treated rats ($100.0 \pm 5.2\%$) (Figure 1C). However, plasma osmolality was not changed following 1 week of d-FEN treatment ($t(12)=-1.77$; not significant, n.s.) and neither were AVP gene expression levels in the PVN ($t(9)=-0.80$; n.s.) and SON ($t(9)=0.98$; n.s.) (Table 1). In contrast, POMC gene expression levels in the ARC were significantly reduced following one week of d-FEN treatment ($t(9)=2.93$; $P = 0.03$).

The unaffected plasma ACTH ($t(12)=-2.17$; $P = 0.07$), plasma corticosterone ($t(12)=1.51$; n.s) and relative adrenal weights ($t(12)=-1.91$; n.s) indicated unchanged activity of the HPA axis (Table 1).

d-Fenfluramine treatment in ABA rats

Since an effective dose of d-FEN was determined in *ad libitum* fed rats, this dose was used in ABA rats in order to influence the development and propagation of ABA. All rats developed hyperactivity following introduction of scheduled feeding. Substantial RWA was observed at the end of the light phase (ZT9–ZT12), which is referred to as food-anticipatory activity (FAA). However, FAA was not affected by d-FEN treatment (day $F(6,84)=15.76$; $P < 0.001$, day $\times$ treatment $F(6,84)=0.27$; n.s). Total daily RWA (day $F(6,84)=32.34$; $P < 0.001$, day $\times$ treatment $F(6,84)=0.51$; n.s) was also not influenced by d-FEN treatment. In addition, cumulative FAA (day 0–6) ($t(14)=1.26$; n.s) and cumulative daily

Figure 1 d-Fenfluramine treatment in *ad libitum* fed rats. (A) Daily food intake, (B) daily relative body weight (% of day –1) and (C) daily water intake in *ad libitum* fed rats treated with vehicle (closed/black, $n=8$) or d-fenfluramine (open/white, $n=6$). Small panels in A and C represent cumulative values. GLM repeated measurements or *t*-tests. * indicates significantly different from vehicle, $P < 0.05$.

RWA (day 0–6) ($t(14)=0.99$; n.s) were not altered by d-FEN treatment (Figure 2A+B). Daily LMA, as measured by telemetric recordings, was not affected by d-FEN treatment over time (day $F(6,78)=11.66$; $P < 0.001$, $F(6,78)=0.20$; n.s), nor was daily cumulative LMA (day 0–6) ($t(13)=0.48$; n.s) (data not shown).

Food intake of ABA rats was not affected by one week of d-FEN treatment (day $F(5,70)=53.05$; $P < 0.001$, day

Table 1 d-Fenfluramine treatment in *ad libitum* fed rats: peripheral and central parameters

	Vehicle	d-FEN
Osmolality (mOsm/kg)	305.0±3.1	330.2±13.9
Leptin (ng/ml)	2.6±0.2	1.7±0.2*
ACTH (pg/ml)	62.4±6.1	101.7±17.1
Corticosterone (µg/dl)	23.7±3.6	16.7±2.2
Adrenal weight (% bodyweight day 6)	0.026±0.001	0.029±0.002
AVP–SON (arbitrary units)	100.0±7.7	91.8±4.1
AVP–PVN (arbitrary units)	100.0±4.0	83.6±2.4
POMC–ARC (arbitrary units)	100.0±6.8	76.9±2.7*

Plasma osmolality, plasma leptin, plasma adrenocorticotrophic hormone (ACTH), plasma corticosterone levels, relative adrenal weight and gene expression levels (arbitrary units) of arginine-vasopressin (AVP) (analyzed in the supraoptic nucleus (SON) and paraventricular nucleus (PVN)), and pro-opiomelanocortin (POMC) (analyzed in arcuate nucleus (ARC)), in *ad libitum* fed rats treated with vehicle ($n=8$) or d-fenfluramine ($n=6$). T-tests, *indicates significantly different from vehicle, $P<0.05$.

× treatment $F(5,70)=0.30$; n.s.). In addition, cumulative food intake (day 1–6) was not different among d-FEN-treated and vehicle-treated ABA rats ($t(14)=1.66$; n.s.) (Figure 2C).

Similar to food intake and RWA, relative body weight was unaffected following one week of d-FEN treatment (day $F(6,84)=383.35$; $P<0.001$, day × treatment $F(6,84)=1.55$; n.s.) (Figure 2D). The final relative body weight of d-FEN-treated ABA rats was not different from vehicle-treated ABA rats ($t(14)=0.90$; n.s.) and plasma leptin levels were below detection limits (<0.5 ng/ml) in both ABA groups.

The disbalance between energy intake and expenditure in ABA rats resulted in severe hypothermia. However, basal body temperature, measured during inactivity in the early light phase (ZT0–ZT3), was not affected by one week of d-FEN treatment (day $F(6,84)=28.35$; $P<0.001$), day × treatment $F(6,84)=0.27$; n.s.) (Figure 2E).

Activation of the HPA-axis, as frequently seen in ABA rats, was not affected by d-FEN treatment. Plasma ACTH levels ($t(13)=0.62$; n.s.), plasma corticosterone ($t(13)=0.87$; n.s.) levels and relative adrenal weight ($t(14)=0.49$; n.s.) were not different between d-FEN-treated rats and vehicle-treated rats following one week of treatment (Table 2).

Thus, d-FEN treatment did not influence any of the characteristics of ABA rats mentioned before. However, d-FEN treatment significantly affected water consumption (day ($F(6,78)=3.20$; $P=0.03$, day × treatment $F(6,78)=2.63$; $P=0.05$). Cumulative daily water intake (day 0–6) was dramatically reduced in d-FEN-treated rats ($58.4 \pm 3.4\%$) as compared with vehicle-treated rats ($100.0 \pm 7.3\%$) ($t(13)=0.49$; $P<0.001$) (Figure 2F). This corresponded with increased plasma osmolality following one week of d-FEN treatment in ABA rats ($t(13)=-2.27$; $P=0.04$) (Table 2). In addition, gene

expression levels of AVP were increased in the SON ($t(9)=-2.30$; $P=0.04$) and in the PVN ($t(9)=-2.71$; $P=0.03$) following one week of d-FEN treatment. In contrast, POMC gene expression in the ARC was not affected by d-FEN treatment ($t(12)=1.23$; n.s.) (Table 2).

Discussion

We showed that chronic d-FEN treatment did not change food intake and RWA in ABA rats. Body weight loss, HPA axis activation and hypothermia were also not affected by d-FEN treatment. However, d-FEN treatment significantly reduced water intake in ABA rats.

The absence of hypophagia in d-FEN-treated ABA rats was unexpected, since it was reported earlier that (one week of) d-FEN treatment reduced food intake in rats (Vickers *et al.* 2000, Rieg *et al.* 1994). This discrepancy might be explained by differences in experimental setup of the current and previous studies, such as duration of food access (1 h vs 1.5 h), running wheel access during feeding (access vs no access), gender (female vs male), age of the rats (± 12 weeks vs 9 weeks) and strain of the rats (Wistar vs Sprague Dawley). Moreover, the experimental approach of the two ABA studies was different; while we examined the effects of d-FEN treatment on development and propagation of ABA during one week of treatment, Rieg *et al.* (1994) focussed the analysis on the day when 25% body weight loss was reached.

Whilst our study was being completed, another study was published, which showed that daily injections of d-FEN enhanced ABA (Atchley & Eckel 2005). This was caused by decreased food intake as well as by additional effects on energy expenditure, most likely being increased brown adipose tissue activity and increased

Figure 2 d-Fenfluramine treatment in ABA rats. (A) Total daily running wheel activity, (B) daily running wheel activity during 4 h preceding food access (FAA), (C) daily food intake, (D) daily relative body weight (% of day -1), (E) daily body temperature during rest and (F) daily water intake in ABA rats treated with vehicle (closed/black, $n=8$) or d-fenfluramine (open/white, $n=8$). Small panels in A-C, F represent cumulative values. GLM repeated measurements or t -tests. * indicates significantly different from vehicle, $P<0.05$.

lipid oxidation. In the present experiment, we found no evidence for anorexic effects of d-FEN in ABA rats. However, d-FEN-treated *ad libitum* fed rats reduced food intake showing efficacy of the dose (10 mg/kg/day) and treatment method (osmotic minipumps) used. Hence, the present results suggest that d-FEN-induced appetite suppression is overruled by physiological signals of starvation in ABA rats. Differences between the current study and the study of Atchley & Eckel (2005) might be explained by differences in experimental setup, such as

the method of administration of d-FEN (osmotic minipump vs daily injections), dosage (10 mg/kg/day vs 0.5 mg/kg/day), duration of food access (1 h vs 2 h), strain of the rats (Wistar vs Long Evans) and possibly the stage of the oestrous cycle at the start of treatment. In an additional experiment, we treated ABA rats with a lower dose of d-FEN (1 mg/kg/day by osmotic minipump) for one week. However, we found that this dose of d-FEN did not affect any parameter of ABA and also did not reduce food intake in *ad libitum* fed rats (data not shown).

Table 2 d-Fenfluramine treatment in activity-based anorexia rats: peripheral and central parameters

	Vehicle	d-FEN
Osmolality (mOsm/kg)	309.8±7.1	340.6±11.3*
Leptin (ng/ml)	n.d.	n.d.
ACTH (pg/ml)	147.5±21.0	133.3±8.6
Corticosterone (µg/dl)	33.7±3.7	29.3±3.0
Adrenal weight (% bodyweight day 6)	0.043±0.004	0.040±0.002
AVP–SON (arbitrary units)	100.0±16.3	161.3±21.8
AVP–PVN (arbitrary units)	100.0±5.8	184.8±27.8*
POMC–ARC (arbitrary units)	100.0±15.3	78.3±8.7

Plasma osmolality, plasma leptin, plasma adrenocorticotrophic hormone (ACTH), plasma corticosterone levels, relative adrenal weight and gene expression levels (arbitrary units) of arginine-vasopressin (AVP) (analyzed in the supraoptic nucleus (SON) and paraventricular nucleus (PVN)), and pro-opiomelanocortin (POMC) (analyzed in arcuate nucleus (ARC)), in ABA rats treated with vehicle ($n=8$) or d-fenfluramine ($n=8$). T-tests, *indicates significantly different from vehicle, $P<0.05$. n.d., not detectable.

In the present study, we observed no effect of chronic d-FEN treatment on RWA or LMA in ABA rats. Total daily activity, activity during the dark phase (data not shown) as well as FAA, were not affected by d-FEN treatment. This corresponds with the two earlier studies, although in these studies total RWA was examined, and not specifically FAA (Rieg *et al.* 1994, Atchley & Eckel 2005). Moreover, effects of d-FEN on RWA, especially on FAA, could have been easily missed by Atchley & Eckel (2005) due to the timing of the d-FEN injections. Previously others reported reduced LMA and RWA in rats treated with d-FEN or other 5-HT_{2C} preferring agonists during positive and negative energy balance (Aulakh *et al.* 1988, Kennett & Curzon 1988, Wilckens *et al.* 1992). Chronic fluoxetine (a SSRI) treatment also reduced RWA, whereas chronic parachlorophenylalanine (a tryptophane hydroxylase inhibitor) treatment increased RWA in semi-starved (SIH) rats (Altemus *et al.* 1996). From the aforementioned, it could thus be hypothesized that d-FEN treatment reduces wheel running in ABA rats. However, d-FEN treatment activates 5-HT_{2C} receptors, that are located on POMC neurons (Heisler *et al.* 2002) and we showed before that chronic α -MSH infusion increased wheel running, specifically FAA, in ABA rats (Hillebrand *et al.* 2005b). These data suggest that d-FEN treatment would increase wheel running in ABA rats. Altogether, unaffected wheel running following d-FEN treatment in the present as well as previous ABA experiments suggest the existence of compensating mechanisms.

Activation of the MC system following d-FEN treatment could not be confirmed. D-FEN treatment did not lead to a reduction in food intake or increased FAA as was observed following α -MSH treatment. Moreover, d-FEN treatment did not increase (but tended to decrease) POMC mRNA expression after one week of treatment. Absence of early effects of chronic d-FEN

treatment on development of ABA might be explained by already over-active POMC neurons during early days of ABA. We recently found transient up-regulation of POMC gene expression during early days of ABA (Hillebrand *et al.* 2005c). Following a few days of food restriction, compensating signals associated with severe negative energy balance could possibly override the putative d-FEN-induced stimulation of POMC neurons. In fact, in *ad libitum* fed rats a significant down-regulation of POMC was observed, which can be explained as a counter-regulatory response to a decrease in food intake and body weight. From this study it thus becomes clear that effects of acute and chronic treatment of d-FEN on e.g. POMC expression levels and food intake are not easily comparable (Choi *et al.* 2002, Heisler *et al.* 2002). Further studies should therefore be aimed at further analyzing actual 5-HT levels and 5-HT_{2C}R binding on POMC neurons during development of ABA following chronic d-FEN treatment.

In the present study, water intake was measured to investigate specificity of d-FEN-induced effects on ingestive behaviour. Total daily water intake was reduced in ABA rats during the whole treatment period. As a consequence, plasma osmolality and mRNA levels of AVP, a neuropeptide involved in regulation of extra cellular fluid balance, were increased after 1 week of treatment. *Ad libitum* fed rats also showed a reduction of water intake following d-FEN treatment. However, this reduction was smaller than observed in ABA rats, and plasma osmolality and AVP gene expression were not changed following 1 week of treatment. Since rats often combine food and water intake, the reduction of water intake in ABA rats may be exaggerated as compared with *ad libitum* fed rats which eat more.

Strong and long-lasting effects of d-FEN treatment on water intake have not been reported before. A modest decrease in water intake was observed following acute

injections of d-FEN, and during early days of long-term d-FEN treatment (Fletcher 1988, Roth & Rowland 1998, Vickers *et al.* 2003), and seems to be mediated by 5-HT_{2C} or 5-HT_{1B} receptors (Clifton *et al.* 2000, Lee *et al.* 2002). The MC system seems not to be involved in the effect of d-FEN on water intake; chronic intracerebroventricular (icv) α -MSH treatment does not lead to reduced water intake of ABA rats (J J G Hillebrand, M J H Kas, R A H Adan, 2005).

D-FEN-induced changes in AVP gene expression have not been described before, whereas d-FEN-induced increases in plasma AVP levels are generally known (Iovino & Steardo 1985, Max 1994, Saydoff *et al.* 1996). Regarding plasma hyperosmolality (van Tol *et al.* 1987, Amaya *et al.* 1999) in ABA rats and the only modelist activation of c-fos in AVP neurons following d-FEN treatment (Javed *et al.* 1999, Mikkelsen *et al.* 1999), the up-regulation of AVP expression is probably an indirect result of hypodipsia and not of a direct effect of d-FEN on AVP neurons. Nevertheless there are also indications that plasma AVP levels are at least partly mediated via 5-HT_{2C}R and the angiotensin system (Saydoff *et al.* 1996). Increased AVP release by the pituitary following acute d-FEN or 5-HT treatment can be reduced by inhibiting angiotensin II production or by antagonizing angiotensin I or 5-HT_{2AC} receptors. In the present experiment we did not measure AVP release (but central AVP expression) and used chronic d-FEN treatment instead of acute treatment (which is not easily comparable), therefore, we consider increased AVP expression as a secondary response to hypodipsia, which is observed to a greater extent in ABA rats than in *ad libitum* fed rats.

We observed no changes in 1 h food intake, RWA, body weight loss or HPA axis activation in d-FEN-treated ABA rats. Strikingly, these three important parameters of ABA are influenced by chronic α -MSH treatment. In addition, POMC mRNA expression levels in ABA rats were not increased (but tended to be decreased) following one week of d-FEN treatment. A prolonged decrease in water intake was observed in d-FEN-treated ABA rats, which corresponded with increased plasma osmolality and increased AVP gene expression. d-FEN was an effective appetite reducing agent for treating obesity. However, it was withdrawn from the market in 1997 because of potential harmful side effects, such as pulmonary hypertension and unusual valvular morphology and regurgitation (Guy-Grand 1995, Connolly *et al.* 1997, MacLean 1999). Yet no information is available on its effects on fluid intake, although patients often complained of a dry mouth and drowsiness (Connolly *et al.* 1997, Bray & Greenway 1999). We conclude that d-FEN treatment results in a strong and prolonged reduction of water intake in food-restricted running rats, but does not enhance the development and propagation of ABA in this study.

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