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Alcohol-preferring AA rats show a derangement in their central melanocortin signalling system

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Abstract

The AA (Alko, Alcohol) rats are selectively bred for their preference of alcohol to water, contrasting to ANA rats that avoid alcohol. They also exhibit a lower growth rate compared to ANA rats, as well as differences in their response to substances affecting food intake. The melanocortin (MC) system is involved in the regulation of feeding behaviour and in mechanisms underlying drug addiction and tolerance. Recently, administration of an MC receptor agonist proved to reduce alcohol intake in AA rats. We predicted that the ratio of endogenous MC receptor agonists (proopiomelanocortin, POMC) and antagonists (agouti-related protein, AgRP) would differ from ANA rats, and that subsequent differences in MC receptor levels would be detectable. We used in situ hybridization to detect an increased ratio of POMC/AgRP mRNA in the arcuate nucleus (Arc) of AA rats. Receptor autoradiography indicated that MC_3 receptor binding differed in the nucleus accumbens and several hypothalamic nuclei, possibly reflecting differences in MC peptide transmission in the AA rats. Our results support the claim that AA rats have a high ratio of POMC/AgRP expression, and that this observation is accompanied by differences in MC_3 receptor levels. © 2002 Published by Elsevier Science Inc.

Keywords: Proopiomelanocortin; Agouti-related protein; Melanocortin receptor autoradiography; Alcohol-preferring rats

1. Introduction

The AA (Alko, Alcohol) rats are selectively bred for their preference of alcohol to water. Apart from alcohol intake, several behavioural and physiological differences have been observed between AA and their controls, the ANA (Alko, Non-Alcohol) rats (Sinclair et al., 1989). Quite interesting is that AA rats tend to be smaller in size. They also exhibit differences in central levels of monoamines, e.g. serotonin and dopamine (Korpi et al., 1991), both of which are involved in the regulation of feeding (Meguid et al., 2000). The α_2-agonist clonidine is a less potent stimulator of food intake in AA rats than in ANA rats, indicating that the two lines differ in their regulation of food intake (Korpi et al., 1991).

There is increasing evidence for the existence of a link between eating disorders, such as anorexia nervosa, and substance abuse, such as alcoholism. Not only do they display similar symptoms (e.g. tolerance, withdrawal, loss of control and social decline) (Beresford and Hall, 1989) but anorexia nervosa in humans is also often associated with abuse of drugs, including alcohol, nicotine and illicit street drugs (Zweben, 1987). Moreover, susceptibility to acohol consumption is a characteristic of rats subjected to food restriction, and this effect cannot solely be attributed to the caloric content of alcohol (Carroll and Meisch, 1984). It is thus possible that the genetic component(s) responsible for the lower body weight in AA rats are responsible also for the alcohol consumption for which these animals have been bred. As AA rats appear to show symptoms of anorexia and are susceptible to alcohol consumption, they are of particular interest as a model to study the connection between anorexia nervosa and alcohol abuse.

One candidate to the proposed link between eating disorders and substance abuse is the melanocortin (MC) system. MC peptides are well known for their involvement in the regulation of feeding behaviour (Spiegelman and Flier, 2001). The MC system is also believed to interfere with the mechanisms underlying drug addiction and tolerance (Alvaro et al., 1997). Moreover, the MC receptor agonist Melanotan II (MTII) was recently shown to reduce

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ethanol intake in AA rats with established ethanol drinking (Ploj et al., 2000).

Five subtypes of the MC receptors (MC_1–MC_5) have been cloned; in the brain, the MC_3 and MC_4 subtypes dominate (Adan and Gispen, 1997). The endogenous MC receptor agonists (α-, β- and γ-MSHs, and ACTH) are derived from the prohormone proopiomelanocortin (POMC), which is expressed in the arcuate nucleus (Arc) in the hypothalamus and the nucleus of the solitary tract (NTS) in the brainstem (O'Donohue and Dorsa, 1982). Other neurons in the Arc express agouti-related protein (AgRP), which is an endogenous MC receptor antagonist known to be involved in the regulation of feeding behaviour (Wikberg et al., 2000).

The low body weight of the AA rats, their difference in regulation of food intake and susceptibility to alcohol consumption led us to hypothesize that they had a difference in the ratio of POMC/AgRP biosynthesis (i.e. a high POMC synthesis relative to AgRP) compared to ANA rats. We also expected that the different expression and subsequent release of POMC/AgRP peptides would result in detectable deviations in MC receptor levels in regions involved in food intake and reward. The hypothesis was tested by quantification of POMC and AgRP mRNA and MC receptor binding in alcohol-naive male AA and ANA rats. In the course of the study, Marinelli et al. (2000) reported that the expression of POMC in the Arc was elevated in AA rats further strengthening our hypothesis.

2. Materials and methods

2.1. Animals

Eight AA and eight ANA rats (187 ± 6 and 221 ± 9 g, respectively, $P < .05$) of the F81 generation were brought from the National Public Health Institute, Helsinki, Finland. The animals were housed in groups of four per cage in air-conditioned rooms (lights on 06:00–18:00 h) at 22–23 °C and a humidity of 55%. The animals had free access to water and R36 food pellets (Labfor, Lactamin, Vadstena, Sweden). The animal experiments were approved by the local ethical committee in Uppsala.

2.2. Tissue preparation

The rats were killed by decapitation. The brains were rapidly removed, frozen in chilled (− 20 to − 30 °C) 2-methyl butane and stored at − 70 °C until further processed. The brains were cut with a cryostat at − 19 °C, and 10 μm coronal sections were thaw mounted on gelatin-coated (receptor autoradiography) or polylysine (in situ hybridization) slides, dried with a fan for 60 min at room temperature and stored at − 70 °C. Sections destined for receptor autoradiography were collected from the striatum at bregma + 1.6, from the hypothalamus at bregma − 1.8 and − 2.8 and from the ventral tegmental area (VTA) at bregma − 5.2 mm. Sections destined for in situ hybridization were collected at bregma − 3.8. From each region, 20 consecutive sections were collected. The sections that best corresponded to the regions as depicted in the brain atlas (Paxinos and Watson, 1997) were selected for autoradiography and in situ hybridization.

2.3. Receptor autoradiography

The MC receptor ligand [Nle^4, D-Phe^7]α-MSH (NDP-MSH) was purchased from Neosystem, France, and radioiodinated by the cloramine-T method. The iodinated product, [^{125}I]NDP-MSH, was purified by high-performance liquid chromatography (HPLC) to a specific activity of 2000 Ci/mmol.

For the autoradiographical experiment, sections were thawed, dried with a fan for 30 min and encircled with a PAP-pen. The sections were preincubated with phosphate-buffered saline (PBS; 0.01 M phosphate, 0.15 M NaCl, pH 7.5) for 15 min, followed by binding buffer (Minimum Essential Medium with Earle's salts, 25 mM HEPES, pH 7.0, 0.2% bovine serum albumin, 1 mM 1,10-phenanthroline, 0.5 mg/l leupeptin and 200 mg/l bacitracin) for 30 min. The sections were then incubated for 120 min with 200 μl binding buffer containing 1 nM

Table 1
[^{125}I]NDP-MSH binding measured autoradiografically in different regions of the brain in AA and ANA rats

		Total (pmol/mg)	MC_3 like (pmol/mg)	MC_4 like (pmol/mg)
AcbC	AA	730 ± 52	296 ± 26	142 ± 17
	ANA	732 ± 44	310 ± 9	129 ± 11
AcbSh	AA	1764 ± 105*	646 ± 42*	245 ± 21
	ANA	2205 ± 168	815 ± 44	242 ± 20
Arc	AA	1956 ± 83*	1530 ± 69**	402 ± 29
	ANA	1667 ± 77	1225 ± 57	344 ± 27
CPu	AA	243 ± 18	112 ± 12	60 ± 10
	ANA	227 ± 19	137 ± 13	54 ± 8
LH	AA	755 ± 50	464 ± 17	136 ± 8
	ANA	684 ± 51	407 ± 33	134 ± 13
PVN	AA	2224 ± 101	1547 ± 101*	1009 ± 112
	ANA	2020 ± 113	1005 ± 164	879 ± 82
VMH	AA	2395 ± 114*	2064 ± 92**	731 ± 58*
	ANA	1902 ± 119	1547 ± 72	563 ± 34
VTA	AA	387 ± 24	374 ± 30	124 ± 15
	ANA	405 ± 40	400 ± 40	111 ± 7

Results are expressed in picomoles per milligram wet weight. The binding corresponds to total binding (i.e. [^{125}I]NDP-MSH binding estimated in the absence of MCR subtype selective competitors) and MC_3- and MC_4-like binding ([^{125}I]NDP-MSH binding estimated in the presence of masking concentrations, respectively, of the MCR subtype selective compunds HS014 and γ_1-MSH). (For further details, see Materials and Methods.) Data are presented as means ± S.E.M. Abbreviations: AcbC — nucleus accumbens core; AcbSh — nucleus accumbens shell; Arc — arcuate nucleus; CPu — caudate putamen, LH — lateral hypothalamus; PVN — paraventricular nucleus of the hypothalamus; VMH — ventromedial nucleus of the hypothalamus; VTA — ventral tegmental area.

* $P < .05$ vs. ANA rats ($n = 8$).

** $P < .01$ vs. ANA rats ($n = 8$).

[^{125}I]NDP-MSH alone (total binding) or in the presence of 3 μM nonlabeled NDP-MSH (nonspecific binding), 1 μM γ_1-MSH (for masking of MC_3 receptors) or 10 nM HS014 (for masking of MC_4 receptors). [^{125}I]NDP-MSH has a K_d of approximately 0.3 nM for both the rat MC_3 and MC_4 receptors, and the concentrations of γ_1-MSH and HS014 were based on previously published calculations (Lindblom et al., 1998). All incubations were made with duplicate sections in a humid chamber at room temperature (20 °C). After incubation, the radioligand solution was removed and the sections were washed 3×5 min in cold (4 °C) PBS, dipped in cold deionized water and dried with a fan for 30 min. Labelled sections and plastic standards (Autoradiographic [^{125}I]Microscales; 2.2–160 nCi/mg; Amersham, Stockholm) were placed in X-ray cassettes and exposed to autoradiographic film (Amersham Hyperfilm) at −20 °C.

Exposure time was 7 days. The films were developed manually (Kodak D19, Unifix), and the autoradiograms were digitized using a dia-scanner (DuoScan T1200, Agfa). The optical densities were then converted to femtomoles per milligram wet weight by comparing to a standard curve calculated from the densities of the coexposed standards by using the NIH Image software (NIH Image 1.62, NIMH, Bethesda, MD). Brain regions were identified with a rat brain atlas (Paxinos and Watson, 1997). [^{125}I]NDP-MSH binding was measured in the nucleus accumbens core (AcbC) and shell (AcbSh), the caudate putamen (CPu), the paraventricular nucleus of the hypothalamus (PVN), the lateral hypothalamus (LH), the ventromedial nucleus of the hypothalamus (VMH), the Arc and the VTA.

2.4. In situ hybridization

The oligonucleotides used were synthesized by TIB Molbiol, Berlin, Germany, and were complementary to the nucleotides 72–122 of POMC mRNA (Drouin and Goodman, 1980) and 73–112 of AgRP mRNA (AF206017) (Korner et al., 2000). The oligonucleotides (40 ng) were labelled with 40 μCi [α-^{35}S]dATP (1250 Ci/mmol) using terminal deoxynucleotidyl transferase (60 U) at 37 °C for 90 min and then purified on Nensorb 20 columns (NEN Life Science Products, London, UK). The brain sections were air dried and hybridized with 10^6 cpm labelled probe/ml in a hybridization solution containing 50% deionized formamide for 20 h at 42 °C. Control sections were coincubated with 0.5 μg/ml nonlabelled oligonucleotide. After hybridization, the sections were washed 4×15 min in 0.15 M NaCl, 15 mM sodium citrate, pH 7.0, at 56 °C and dehydrated. Labelled sections and plastic standards (Autoradiographic [^{14}C]Microscales; 30–880 nCi/g, Amersham) were placed in X-ray cassettes and exposed to

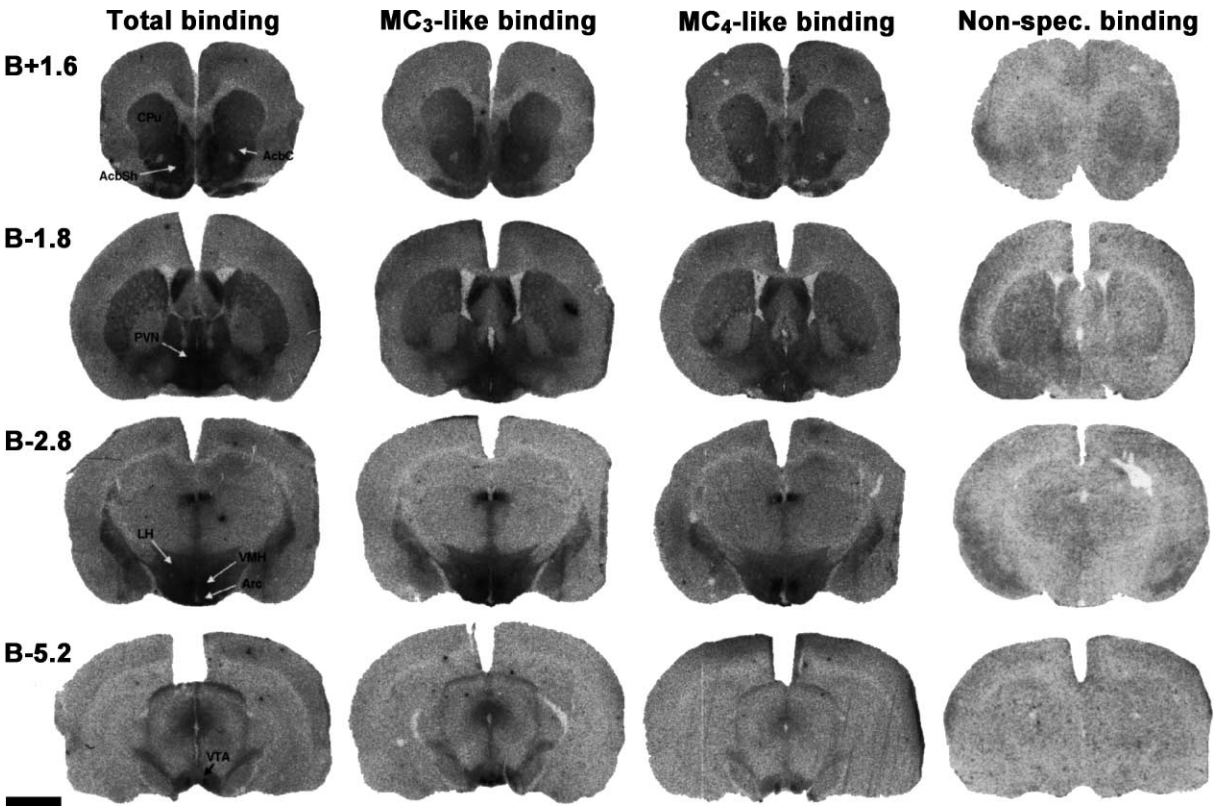

Fig. 1. Representative autoradiograms from one ANA rat showing binding of 1 nM [^{125}I]NDP-MSH alone (total binding), binding in the presence of 10 nM HS014 (MC_3-like binding), binding in the presence of 1 μM γ1-MSH (MC_4-like binding) and binding in the presence of 3 μM NDP-MSH (nonspecific binding) at bregma +1.6, −1.8, −2.8 and −5.2 mm. Scale bar: 3 mm.

autoradiographic film (Hyperfilm-βmax, Amersham). After 6 weeks, the films were developed manually (Kodak D19, Unifix), and the slides were dipped in Kodak NTB-2 emulsion at 43 °C, dried overnight and exposed for another 6 weeks at 4°C, developed and counterstained with Cresyl violet. The autoradiograms were digitized and the optical density converted to becquerels per milligram wet weight in a similar fashion as described above. The photoemulsion dipped sections were digitized with an Axiocam digital camera connected to an Axioplan 2 imager microscope using the AxioVision 3.0 software (all Zeiss, Germany). The labelling in the Arc was then quantified by manually counting the silver grains in that area.

2.5. Statistical analysis

All results are expressed as means ± S.E.M. The receptor autoradiography data were for each region of interest subjected to analysis of variance (ANOVA) for repeated measures followed by Fisher's protected least significant difference (PLSD) test where appropriate. An unpaired Student's *t* test was used to evaluate the in situ hybridization data. $P < .05$ was used as the criterion of statistical significance.

3. Results

3.1. MC receptor binding

Results from the MC receptor binding experiment are shown in Table 1. The levels of MC receptor binding were significantly higher for AA rats compared to ANA rats in the Arc [$F(1, 13) = 9.82$, $P < .01$], PVN [$F(1, 14) = 5.10$, $P < .05$] and VMH [$F(1, 13) = 13.64$, $P < .01$], but lower in the AcbSh [$F(1, 14) = 6.44$, $P < .05$]. No difference in MC receptor binding was found in the AcbC [$F(1, 14) = 0.001$, $P = .98$], CPu [$F(1, 14) = 0.04$, $P = .95$], LH [$F(1, 13) = 1.39$, $P = .26$] and VTA [$F(1, 14) = 0.185$, $P = .67$]. Post hoc analysis indicated that for AA rats, the total binding was higher in the Arc and VMH, but lower in the AcbSh. The post hoc analysis also included estimations of MC_3- and MC_4-like receptors (see Materials and Methods for details). MC_3-receptor-like binding was higher in the Arc, PVN and VMH, but lower in the AcbSh. MC_4-receptor-like binding was higher only in the VMH. Representable autoradiograms are shown in Fig. 1.

Table 2
In situ hybridization of AgRP and POMC in the Arc of AA and ANA rats

	AgRP (Bq/mg)	AgRP (grains)	POMC (Bq/mg)	POMC (grains)
AA	2.87 ± 0.80*	11.3 ± 4.5	3.79 ± 1.13	30.6 ± 5.7*
ANA	6.74 ± 1.88	26.1 ± 8.5	1.71 ± 0.35	14.8 ± 2.2

Results are expressed in becquerel per milligram wet weight and number of silver grains estimated from the densities and grain counts of photoemulsions exposed to the [α-^{35}S] labelled hybridizations probes. Data are presented as means ± S.E.M.

* $P < .05$ vs. ANA rats ($n = 4$).

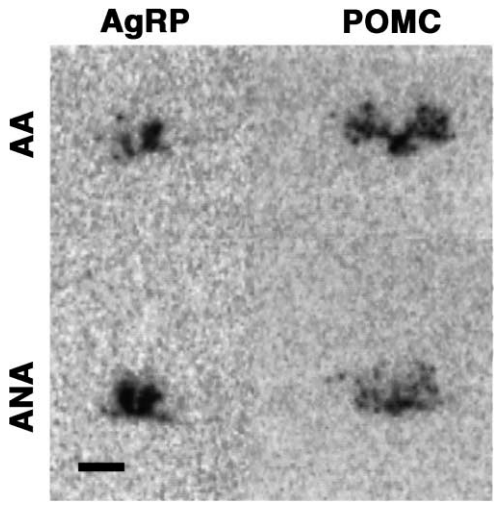

Fig. 2. Representative autoradiograms from AgRP and POMC in situ hybridizations in the ARC of AA and ANA rats. Scale bar: 0.5 mm.

3.2. AgRP and POMC expression

Analysis of the autoradiograms from the in situ hybridization experiment indicated that AgRP mRNA levels in the Arc were lower for AA rats than ANA rats ($P < .05$). POMC levels tended to be higher for AA rats, although the differences did not reach the level of conventional significance ($P = .065$). Analysis of the photoemulsion-treated sections supported these observations. The number of counted grains for the POMC-hybridized sections was significantly higher in AA rats ($P < .05$). For AgRP-hybridized sections, the number of grains appeared to be lower, although the difference did not reach significance ($P = .078$). Data from the in situ hybridization experiment are presented in Table 2. Representable autoradiograms are shown in Fig. 2.

4. Discussion

AA rats are bred for their preference of alcohol to water, but they also exhibit a lower growth rate compared to ANA rats (Sinclair et al., 1989), as well as differences in their response to substances affecting food intake (Korpi et al., 1991). Recently, administration of the MC agonist MTII proved to reduce alcohol intake in AA rats (Ploj et al., 2000). Based on this evidence, we hypothesized that these animals differed from ANA rats in the regulation of the MC system. We predicted that the ratio of hypothalamic POMC/AgRP biosynthesis would differ in AA rats, and that this also might be reflected by differences in MC receptor levels.

As expected, we were able to detect an elevated level of POMC expression in the Arc of AA rats. There was also

evidence for a reduced level of AgRP mRNA in this area, in support for our theory of a higher α-MSH/AgRP ratio in AA rats. Differences in MC_3 receptor expression in the nucleus accumbens (a decrease) and several hypothalamic nuclei involved in the regulation of food intake (increases) were also observed, possibly reflecting differences in MC peptide transmission in the AA rats. AgRP and POMC synthesizing neurons in the Arc coexpress the MC_3 receptor (Bagnol et al., 1999), indicating that it may serve as an autoreceptor. If so, the high levels of MC_3-like binding in the Arc, PVN and VMH — nuclei involved in the regulation of body weight homeostasis (Bagnol et al., 1999) — may reflect an increased transmission of α-MSH in these nuclei of the AA rats.

The finding that administration of MTII may reduce alcohol intake in AA rats (Ploj et al., 2000) suggests that a low level of POMC expression would have been expected. In contrast, we here observed a high level of Arc POMC mRNA. However, there may be considerable differences between an acute administration of a synthetic MSH analogue and the transmission of endogenous MC peptides. The timing of administration, dose levels at the site of action and the stability of the peptides are important issues. Moreover, the selectivity profile of MTII differs from, e.g., α-MSH (Adan et al., 1999).

Leptin is secreted from adipose tissue in proportion to the amount of adipose mass (Greenberg and Boozer, 1999), functioning as a circulating hormone causing actions on hypothalamic neurons that express leptin receptors. Among the leptin receptor containing neurons are groups of neurons in the Arc that coexpress AgRP or POMC with other signalling peptides, for which, in the normal animal, a positive energy balance causes an increase in POMC and a decrease in AgRP activity, resulting in a reduced food intake. The opposite effect is caused by a negative energy balance. Thus, as a result body weight homeostasis may be maintained (reviewed in Spiegelman and Flier, 2001). Leptin has also been shown to modulate electrical self-stimulation in the LH (Fulton et al., 2000), and some of the leptin effect on food intake may thus be attributed to a decreased reward value of food (Figlewicz and Woods, 2000). The LH is an area believed to be involved in feeding behaviour (Elmquist et al., 1999) and is also considered to have a role in reward mechanisms (McBride et al., 1999). It receives input from the leptin sensitive POMC neurons in the Arc (Elias et al., 1999). As the MC system is thought to be a downstream mediator of leptin actions on food intake (Kask et al., 1998), it is plausible to assume that MC peptides also may mediate the effects of leptin on the reward system. There is also evidence for a role of the MC system in the mechanisms underlying drug addiction and tolerance (Alvaro et al., 1997). MCs thus may constitute a biological link between regulation of energy homeostasis and the brain's reward system.

In several different models of underweight or obesity, the POMC system appears to be affected in one way or another. Arc POMC expression is inhibited by fasting and stimulated by leptin administration (Mizuno et al., 1998; Schwartz et al., 1997). Food restriction and food deprivation is associated with a decrease in Arc POMC expression (Brady et al., 1990). Interestingly, this is also the case for long-term feeding with a high-fat diet resulting in obesity, an observation that may be attributed to leptin resistance (Lin et al., 2000). However, involuntary overfeeding causes increased POMC mRNA levels and a MC-mediated hypophagia (Hagan et al., 1999). Moreover, resistance to diet-induced obesity, as exhibited by A/J mice, is associated with an increase in POMC mRNA (Bergen et al., 1999). AgRP, on the other hand, is reduced after short-term feeding with a high-fat diet (Ziotopoulou et al., 2000), inhibited by leptin and stimulated by fasting (Mizuno and Mobbs, 1999).

Despite the lower body weight of the AA rats, their high levels of POMC mRNA thus resemble what is observed after involuntary overfeeding and resistance to diet-induced obesity. Moreover, the low levels of AgRP mRNA observed in AA rats are similar to short-term models of overfeeding. One explanation for the disturbed pattern of AgRP and POMC expression in AA rats might be an increased response to adiposity signals such as leptin. In fact, a leptin hypersensitivity model of anorexia nervosa have been discussed (Arch et al., 1998).

In summary, our results support the claim that AA rats have a higher ratio of POMC/AgRP expression compared to ANA rats, and that these differences are accompanied by differences in MC_3 receptor levels. Resemblances to effects of involuntary overfeeding and resistance to diet-induced obesity suggest that AA rats may be hypersensitive to adiposity signals, such as leptin, in line with a previously proposed model of anorexia nervosa. These results strengthen the hypothesis that the MC system may constitute a link between the regulation of energy homeostasis and the brain's reward system.

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