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Endogenous ACTH, not only α-melanocyte-stimulating hormone, reduces food intake mediated by hypothalamic mechanisms

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Schulz C, Paulus K, Lobmann R, Dallman M, Lehnert H. Endogenous ACTH, not only α-melanocyte-stimulating hormone, reduces food intake mediated by hypothalamic mechanisms. *Am J Physiol Endocrinol Metab* 298: E237–E244, 2010. First published November 17, 2009; doi:10.1152/ajpendo.00408.2009.—ACTH and α-melanocyte-stimulating hormone (α-MSH) are both consecutively processed from proopiomelanocortin (POMC), which is synthesized in hypothalamic arcuate neurons innervating the paraventricular nuclei (PVN). POMC secretion/synthesis is regulated by energy availability. ACTH and α-MSH bind with equal affinity to melanocortin-4 receptors and elicit similar effects on signal transduction in-vitro. Endogenous α-MSH thus far is believed to be the major physiological agonist and to act in an anorexigenic manner. Until now, it was fully unknown whether endogenous ACTH is also involved in the regulation of appetite and food intake. In this study in rats, we now show that icv ACTH as well as α-MSH possess anorexigenic effects in the PVN or areas in close proximity in vivo and that the effect of ACTH is direct and not mediated via α-MSH. We investigated the roles of endogenous ACTH and α-MSH by PVN application of the respective antibodies under different physiological conditions. In satiated rats with high levels of ACTH and α-MSH in the PVN, antibody administration increased food intake and body weight gain; hungry animals were unaffected. Finally, repeated injections of ACTH antibodies into PVN resulted in persistently increased food intake during the light period. These data now provide robust evidence that endogenous ACTH without further processing acts in the PVN or areas in close proximity to reduce food intake under conditions of feeding-induced satiety.

adrenocorticotropic hormone; appetite; hypothalamus; paraventricular nucleus; melanocortin-4 receptor

THE ARCUATE NUCLEUS (ARC) of the hypothalamus represents one of the most relevant brain areas involved in physiological appetite regulation, integrating both central and peripheral signals to maintain body weight homeostasis (13, 35). An example of this is the melanocortin system, which is crucial in mediating the central effects of the white adipose tissue hormone leptin (43, 46). The ARC contains at least two distinct populations of neurons, proopiomelanocortin (POMC)- and cocaine- and amphetamine-regulated transcript (CART)-expressing, and neuropeptide Y (NPY)- and agouti-related protein (AgRP)-expressing cells. The peptide precursor POMC is consecutively cleaved by proteolytic enzymes into a number of smaller peptides (melanocortins), including ACTH and α-MSH (39). When applied into the central nervous system in pharmacological doses, both inhibit food intake through melanocortin-4 receptors (MC4R), which are highly expressed in the paraventricular (PVN) and dorsomedial nuclei (DMN) and lateral hypothalamus (LHA) (21, 30). ACTH and α-MSH bind to the MC4R with similar affinity (1) and activate identical signal transduction pathways (15). Melanocortins and the MC4R are critically involved in body weight homeostasis, since mutations in the MC4R (7, 18) or the POMC gene (22) result in obesity in mice and humans, respectively (9).

Fasting reduces mRNA expression (5), synthesis (37), and release of POMC (6), whereas POMC expression is positively regulated by leptin (8). Increased POMC expression in the hypothalamus through local delivery of recombinant adenoassociated virus encoding POMC (rAAV-POMC) diminishes food intake and visceral fat in the genetically obese Zucker rat (25) and reduces both obesity and glucose intolerance in aged rats (26). Furthermore both intracerebroventricular (icv) application of ACTH and its NH_2-terminal fragments [ACTH-(1–10), α-MSH] acutely inhibit feeding in rodents (1, 20, 36); however, it is not clear whether in these experiments ACTH itself was effective or whether proteolytic processing, i.e., into α-MSH, was necessary to elicit the MC4R-mediated effects.

To investigate whether the PVN or areas in close proximity are important or even major targets of icv melanocortins, we examined the actions of icv α-MSH and ACTH in combination with local immunological blockade by antibodies (Ab) administered into the PVN. Furthermore, to elucidate whether proteolytic processing to α-MSH is required for the anorexigenic properties of ACTH icv, we administered the peptide together with α-MSH Ab, which was applied locally into the PVN. Since the Ab has no significant cross-reactivity with ACTH, it would block the anorexigenic actions of ACTH only if processing into α-MSH was required and exerted.

Both α-MSH and ACTH are present in the hypothalamus, but the exact amounts and thus their relative quantities are difficult to determine and are a matter of debate (29, 35, 37). Currently, α-MSH is discussed as the MC4R ligand of major importance. However, this has not been supported by experimental evidence, and other potential ligands, i.e., POMC and ACTH, are also secreted from the ARC (37). To elucidate the role of endogenous ACTH at the MC4R, we performed a series of experiments comparing the efficacy of blocking the action of endogenous ACTH and α-MSH in the PVN area on food intake.

C. Schulz and K. Paulus contributed equally to this paper.

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METHODS

Animals and General Information

Male Wistar rats (weight range 225–250 g at purchase), obtained from Harlan Winkelmann, Borchen, Germany, were employed. Before the experiment, animals were kept in groups of four under a 12:12-h light-dark cycle (light on 6 AM) at 23°C; food pellets (no. 2018; Harlan Winkelmann, Borchen, Germany) and tap water were available ad libitum, if not otherwise stated. After central implantation of cannulas, rats were housed individually. During the experiments, food and water intake and body weights were monitored as specified in figures and tables. Animals were killed by decapitation. Animal numbers are given in figure or table legends.

The experimental protocols for animals and their care were in accordance with German law and were approved by the Committee on Animal Care. All experiments met the highest standards of humane animal care and complied with the Institute for Laboratory Animal Research (ILAR) *Guide for Care and Use of Laboratory Animals*.

Surgical Procedures

For implantation of microinjection units, animals were anesthetized with ketamine (100 mg/kg ip Ketavet; Pharmacia, Karlsruhe, Germany) and 2% xylazine (14 mg/kg im Rompun; Bayer Vital, Leverkusen, Germany) and kept on a regulated heating pad during surgery. For intracerebral implantation, animals were placed in a stereotaxic frame. Microinjection units made from fused silica capillary (150 μm OD) were implanted either in the PVN or in both PVN and lateral ventricle. One unit per site was implanted; in animals with both PVN and icv implantation, units were placed in different hemispheres. Hemispheres were chosen at random. Coordinates for implantation into PVN were as follows: anterior −1.80, lateral ± 0.40, and ventral −7.70 mm, relative to the bregma; and for the lateral ventricle: anterior −0.80, lateral ± 1.40, and ventral −3.60 mm, relative to the bregma. Coordinates were taken from the brain atlas of Paxinos and Watson (34). During postoperative recovery, rats were kept on the heating pad.

The location of the microinjection units was determined to be the point of termination of the cannula track in Nissl-stained sections. All icv units were correctly placed in the ventricle, and the tips of all microinjection units aimed at the PVN were within 0.5 mm of the nucleus.

After surgery, animals were allowed to recover for 1 wk, during which they were handled daily. Food and water intake and the development of body weight were monitored. Injection units were kept patent by daily applications of 1 μl (PVN) or 2.5 μl (icv) of physiological saline over 2 min.

Statistical Evaluation

All values are given as means ± SE. Statistical analysis was accomplished by using SPSS, version 11.0, for Windows. A one-way analysis of variance for repeated measures was performed to analyze for differences among food, water, and body weight gain over the whole observation period. At every time point, Bonferroni's *t*-test was carried out to reveal differences between groups.

Substances and Substance Application

ACTH-(1–39) (rat; A7075), α-MSH (M4135), and IgG (I5006) were purchased from Sigma-Aldrich (Taufkirchen, Germany) and Ab's for ACTH (T-4001) and α-MSH (T-4433) from Bachem (Weil am Rhein, Germany).

Cross-reactivities for the Ab's were as follows: ACTH Ab: mouse, rat, human ACTH-(1–39) 100%; human CLIP [corticotropin-like intermediate lobe peptide, ACTH-(18–39)] 100%; human ACTH (1–24) 1%; human ACTH (7–38) 0%; LHRH 0%; human, mouse, ovine, porcine, rat PACAP-38 0%; rat β-endorphin 0%; α-MSH 0%; α-MSH Ab: α-MSH 100%; human ACTH 0.02%; rat ACTH 1.25%; α-endorphin, human β-endorphin 0%; γ-endorphin 0%; β-MSH 0%; γ_3-MSH 0%.

All substances were diluted in sterile 0.9% saline in a volume of 1 μl for PVN or 2.5 μl for icv applications, respectively. Applications were performed over 2 min.

Experimental Design

Both icv ACTH and α-MSH exert their effects on food intake and body weight in or near the PVN. Animals were equipped with both icv and PVN microinjection units. After recovery, animals were food deprived (free access to water) for 24 h before applications at 9 a.m.

ACTH AB INTO PVN, ACTH ICV. Animals received a PVN application of ACTH Ab (2 μg/rat) or IgG (2 μg/rat); administration of either ACTH (1 nmol/rat) or saline icv was performed 5 min later. Following administration, food was offered to the animals; food and water intake was then measured over 24 h. Body weight was determined at application, at 6 h, and at the end of the observation period. After the experiment, animals were allowed a washout phase of 5 days before the next experiment according to the above scheme was performed. Each animal received three different treatments; treatments were assigned to the experimental animals in a pseudo-random fashion to ensure that all possible combinations of treatments and treatment orders were evenly represented. At the beginning of the first treatment, body weight of the animals was 297.8 ± 7.9 g.

α-MSH AB INTO PVN, α-MSH ICV. Animals received a PVN application of α-MSH Ab (2 μg/rat) or IgG (2 μg/rat); administration of either α-MSH (1 nmol/rat) or saline icv was performed 5 min later. The experimental setting was as described in the experiment above. At the beginning of the first treatment, body weight of the animals was 297.6 ± 7.9 g.

Effects of combined administration of α-MSH Ab in the PVN and ACTH icv on food intake and body weight. As in *Both icv ACTH and α-MSH exert their effects on food intake and body weight in or near the PVN*, animals were equipped with both icv and PVN microinjection units. After recovery, animals were food deprived (free access to water) for 24 h before applications at 9 AM. Either α-MSH Ab (2 μg/rat) or IgG (2 μg/rat) was applied into the PVN; ACTH (1 nmol/rat) or saline was administered icv 5 min later. Following administration, food was offered to the animals; food and water intake was then measured over 24 h. Body weight was determined at application, at 6 h, and at the end of the observation period. After the experiment, animals were allowed a washout phase of 5 days, before the next experiment according to the above scheme was performed. Each animal received three different treatments; treatments were assigned to the experimental animals in a pseudo-random fashion to ensure that all possible combinations of treatments and treatment orders were evenly represented. At the beginning of treatment, body weight of the animals was 294.8 ± 9.9 g.

Effects of endogenous ACTH and α-MSH in or near the PVN on food intake and body weight in satiated and hungry rats. 1) Animals were equipped with PVN microinjection units. To study the relevance of endogenous α-MSH and ACTH in the regulation of food intake, animals were subjected to two different food regimens.

SATIATED RATS. Animals were food deprived (free access to water) for 24 h and then refed at 9 AM to induce a state of evening satiety. This feeding regimen was chosen to allow the same application time and observation period as in HUNGRY RATS (see below) to minimize the effects of circadian rhythms, e.g., feeding and physical activity patterns, hormone levels, etc.

At 8 PM of the same day, animals were injected with either ACTH Ab (2 μg/rat), α-MSH Ab (2 μg/rat), a combination of both Ab's (2 μg/rat for each Ab), or IgG (2 μg/rat) into the PVN, and preweighed food and water were supplied.

HUNGRY RATS. Animals were fed ad libitum during the whole experiment and thus were hungry in the evening at the beginning of

their regular feeding period. At 8 PM, animals received injections as under SATIATED RATS.

As stated in *Effects of combined administration of α-MSH Ab in the PVN and ACTH icv on food intake and body weight*, food and water intake as well as body weight were measured over 24 h; three different treatments were tested in each individual. At the beginning of the first treatment, body weight of the animals was 298.1 ± 9.9 g.

2) Equipment and feeding regimen as well as PVN applications were as stated in (*1*) above. However, animals were treated only once, and food and water intake were only monitored over 10 h. Body weight was determined at application and at the end of the observation period. At the beginning of treatment, body weight of the animals was 299.1 ± 8.5 g.

Subchronic blockade of endogenous ACTH in the PVN and areas in close proximity. Animals were equipped with a microinjection unit in the PVN. After recovery, rats were treated twice daily (8 AM and 6 PM) with ACTH Ab (2 μg/rat), or IgG (2 μg/rat) as control. At times of injection, food and water intake were monitored and day- and nighttime food intakes were calculated to monitor separately the effects of substance application in satiated animals (i.e., during the day) and hungry animals (i.e., at night), respectively. Body weight was measured following the morning application. During the experiment, animals had free access to food and water. At the beginning of treatment, body weight of the animals was 274.7 ± 12.6 g.

RESULTS

Both icv ACTH and α-MSH Exert Their Effects on Food Intake and Body Weight in or Near the PVN

ACTH ab into PVN, ACTH icv. The application of the different substances significantly affected cumulative food intake in 24-h-starved animals ($F_{3.16} = 17.915$, $P < 0.01$). The icv injection of ACTH significantly reduced cumulative food intake over the observation period compared with the saline/IgG group. The injection of ACTH Ab into the PVN abolished the anorexigenic effect of ACTH (Fig. 1*A*; statistical comparisons in figure). There were no significant differences between the application of ACTH/ACTH Ab and saline/IgG. The results for water intake were similar to those for food intake ($F_{3.16} = 8.085$, $P < 0.01$): the PVN application of ACTH Ab abolished the effects of icv ACTH on water intake (data not shown); ACTH/ACTH Ab and saline/IgG effects on water intake did not differ significantly. Body weight gain was not significantly affected by treatments ($F_{3.16} = 1.902$, $P = 0.170$).

α-MSH ab into PVN, α-MSH icv. Treatments had a significant effect on food intake and the profile was similar to the experiment employing ACTH and the respective Ab ($F_{3.20} = 7.242$, $P < 0.05$). α-MSH exerted a significant anorexigenic effect from 1 h after injection until 3 h. The additional injection of α-MSH Ab into the PVN prevented the anorexigenic effect of α-MSH (Fig. 1*B*; statistical comparisons in figure). The results for water intake resembled those for food intake ($F_{3.20} = 3.237$, $P < 0.05$): the application of α-MSH Ab into the PVN abolished the effects of icv α-MSH on water intake (data not shown). Water intake in α-MSH/α-MSH Ab-treated animals did not differ from that of the saline/IgG group. Body weight gain was affected by treatments as a trend ($F_{3.20} = 3.049$, $P = 0.052$).

The results of these experiments clearly show that the effects of both icv ACTH and α-MSH are mediated by targets in or in close proximity to the PVN. Furthermore, they demonstrate the in vivo efficacy of the selected ACTH and α-MSH Ab.

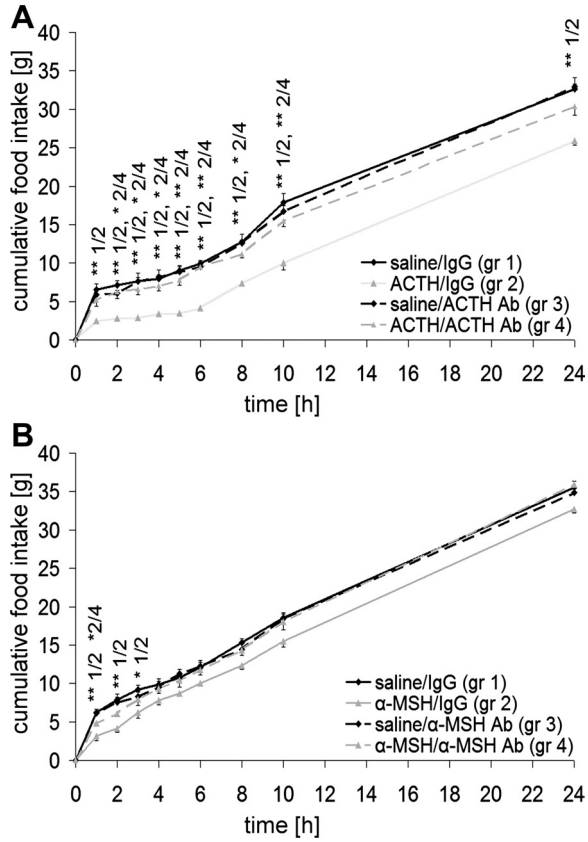

Fig. 1. Food intake after intracerebroventricular (icv) ACTH or α-MSH and the respective antibody (Ab) into the paraventricular nucleus (PVN). Cumulative food intake was measured in 24-h food-deprived male Wistar rats after a single icv dose of ACTH or α-MSH and the respective Ab into the PVN. *Significant differences in unifactorial ANOVA; 1/2 and 2/4 indicate significant differences between groups. All values are given as means ± SE. *A*: saline or ACTH icv and ACTH Ab or IgG into the PVN [$n = 4–6$ per group (gr)]. *B*: saline or α-MSH icv and α-MSH Ab or IgG into the PVN ($n = 6$ per group).

Effects of Combined Administration of α-MSH Ab in the PVN and ACTH icv on Food Intake and Body Weight

Treatments significantly affected cumulative food intake in 24-h-starved animals ($F_{3.20} = 36.023$, $P < 0.01$). Infusion icv of ACTH significantly decreased cumulative food intake in rats that received α-MSH Ab into the PVN and ACTH icv, and food intake was as low as in the group treated with ACTH icv and IgG into the PVN. Application of α-MSH Ab into the PVN of animals that received saline icv did not affect food intake (Fig. 2; statistical comparisons in figure). Water intake, primarily prandial, was similarly reduced in rats that ate less ($F_{3.20} = 17.946$, $P < 0.01$): the PVN application of α-MSH Ab did not abolish the effects of icv ACTH on water intake (data not shown). Body weight gain was significantly affected by treatments ($F_{3.20} = 18.076$, $P < 0.01$). ACTH icv and IgG (PVN) significantly reduced body weight gain after 6 h; the same was observed when α-MSH Ab (PVN) was applied in combination with ACTH icv. These effects persisted at 24 h.

The finding that the application of α-MSH Ab did not affect the anorexigenic effect of ACTH clearly shows that ACTH does not have to be processed to α-MSH to affect food intake.

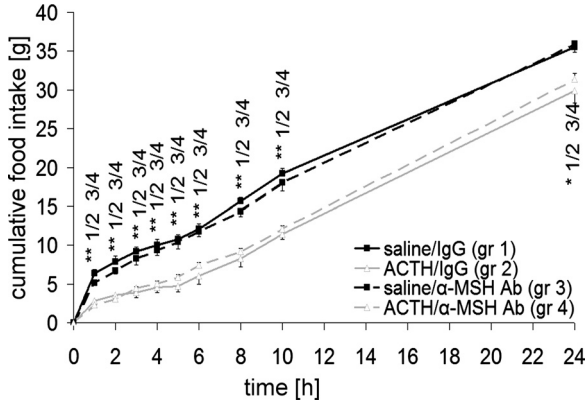

Fig. 2. Food intake after icv saline or ACTH and α-MSH Ab or IgG into the PVN. Cumulative food intake was measured in 24-h food-deprived male Wistar rats after a single icv dose of saline or ACTH and α-MSH Ab or IgG into the PVN (n = 4–6 per group). *Significant differences in unifactorial ANOVA; 1/2 and 3/4 indicate significant differences between groups. All values are given as means ± SE.

Effects of Endogenous ACTH and α-MSH in or Near the PVN on Food Intake and Body Weight in Satiated and Hungry Rats

All animals were injected in the evening, when animals normally are hungry and commence their daily food intake cycle. To induce satiation at this time of day, rats were starved for 24 h and then allowed food ad libitum from the morning on the day of study. In satiated animals, cumulative food intake was significantly affected by treatments ($F_{3.32}$ = 12.247, P < 0.01). Injection of either ACTH Ab or α-MSH Ab into the PVN significantly increased cumulative food intake compared with IgG-treated animals; the combined application of both Ab's did not increase food intake further (Fig. 3*A*; statistical comparisons in figure). In contrast to these findings, in hungry rats, the application of Ab's did not affect cumulative food intake ($F_{3.33}$ = 0.661, P = 0.582; Fig. 3*B*).

The results for cumulative water intake in satiated rats were similar to those for food intake in these animals ($F_{3.32}$ = 8.584, P < 0.01): ACTH Ab, α-MSH Ab, and the combination of both Ab's applied to the PVN significantly increased cumulative water intake compared with IgG-treated animals (data not shown). In contrast to these findings, Ab applications to the PVN were without effect on cumulative water intake in hungry rats ($F_{3.33}$ = 0.787, P = 0.510; data not shown).

In satiated rats, body weight gain was significantly affected by treatments ($F_{3.32}$ = 3.357, P < 0.05). ACTH Ab application into the PVN significantly increased body weight gain after 24 h; the same effect was observed when α-MSH Ab was applied alone or in combination with ACTH Ab (Fig. 3*C*). In contrast to these findings, body weight gain was not affected by central PVN Ab treatments in hungry animals ($F_{3.33}$ = 0.263, P = 0.852; Fig. 3*D*).

Repetition of this experiment employing a single application of substances showed that cumulative food and water intake

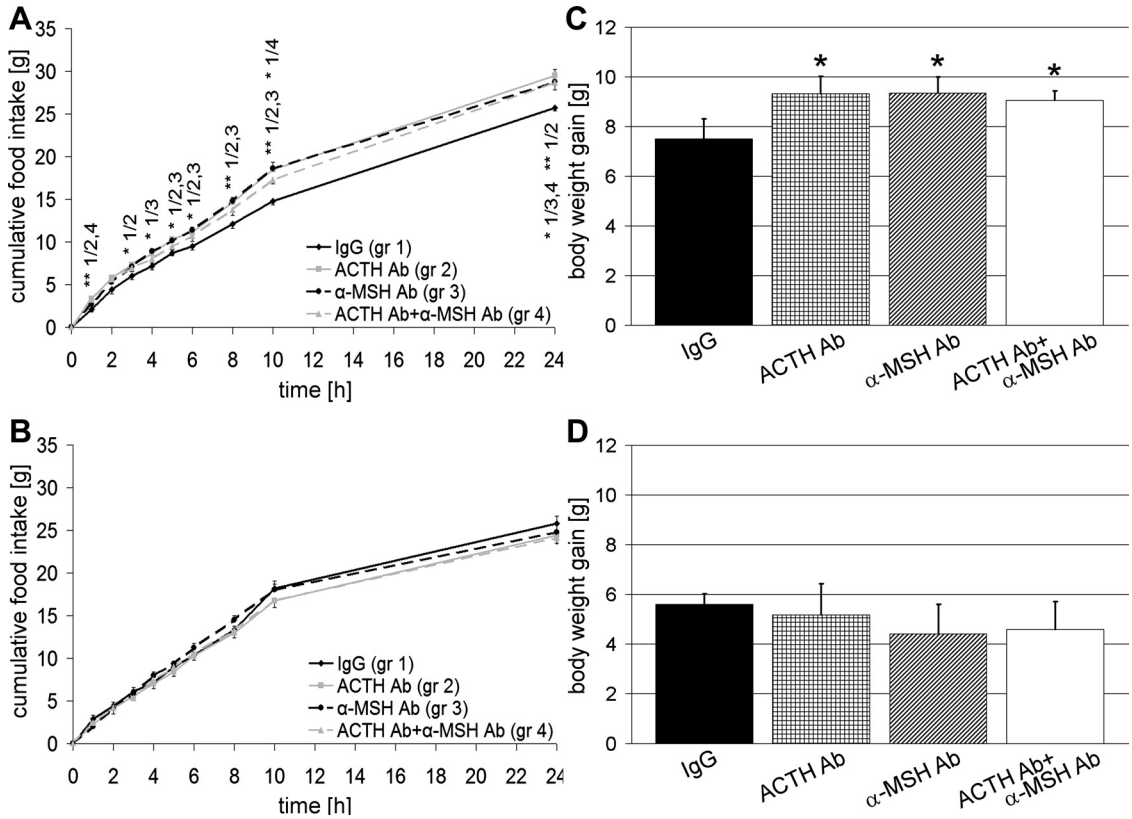

Fig. 3. Food intake and body weight gain in satiated and hungry male Wistar rats after ACTH Ab and/or α-MSH Ab into the PVN. All values are given as means ± SE. *A*: cumulative food intake in satiated rats (n = 9–10 per group). *Significant differences in unifactorial ANOVA; 1/2 and 3/4 indicate significant differences between groups. *B*: cumulative food intake in hungry rats (n = 10 per group). *C*: body weight gain in satiated rats (n = 9–10 per group). *Significant difference between treatment group and control (IgG). *D*: body weight gain in hungry rats (n = 10 per group).

and body weight gain in both satiated and hungry rats closely matched the above results (see online supplemental material).

The results from these experiments clearly show that, in satiated rats, both endogenous ACTH and α-MSH possess anorexigenic effects.

Subchronic Blockade of Endogenous ACTH in the PVN and Areas in Close Proximity

Rats fed ad libitum were injected with ACTH Ab into the PVN twice daily. Over the observation period of 7 days, the daytime (8 AM - 6 PM) cumulated food intake was significantly affected by treatments ($F_{1.12} = 38.951$, $P < 0.01$). ACTH Ab treatment significantly increased cumulative food intake compared with controls (Table 1). This increase in daytime cumulative food intake was significant on each experimental day (Table 1). Statistical evaluation of nighttime food intake (interval 6 PM - 8 AM) revealed that cumulated food intake was increased in ACTH Ab-treated animals compared with the IgG group ($F_{1.12} = 9.062$, $P < 0.05$); however, individual daily differences in food intake were not statistically significant (Table 1). Body weight gain did not differ significantly over the observation period ($F_{1.12} = 0.007$, $P = 0.934$). The effect of ACTH Ab on water intake was similar to that on food intake; again, the differences in water intake were significant only for measurements during the daytime interval (day: $F_{1.12} = 40.181$, $P < 0.01$; night: $F_{1.12} = 0.294$, $P = 0.597$; data not shown).

DISCUSSION

Several experiments have shown that the central application of ACTH affects food intake (17); however, it is not known whether this is a direct effect of ACTH itself or whether proteolytic cleavage, i.e., into α-MSH, is required. Physiologically, in the hypothalamus, endogenous POMC is cleaved by prohormone convertase (PC)1 into ACTH, consisting of the 39 NH_2-terminal amino acids of POMC [thus often termed ACTH-(1–39)]. ACTH is then proteolytically cleaved by PC2 into ACTH-(1–17) and CLIP. From ACTH-(1–17), mature α-MSH is generated via desacetyl α-MSH, mediated by carboxypeptidase E, peptidylglycyl-α-amidating monooxygenase (PAM), and *N*-acetyltransferase (39). The prohormone convertases PC1 and PC2 are localized in dense core secretory granules (41), indicating that their cleavage products are secreted in a regulated fashion and not via the constitutive pathway. It was shown that not only α-MSH but also ACTH and POMC are effectively secreted by neurons of the PVN (37).

The generation of both ACTH and α-MSH from the same peptide precursor raises experimental difficulties in studying distinct roles of these peptides in body weight homeostasis. Approaches such as knockout models for POMC, siRNA, or antisense oligonucleotide techniques are, for obvious reasons, not useful to study the distinctive effects of the two peptides. Furthermore, since both ACTH and α-MSH effectively bind to the MC4R, this prevents the use of receptor antagonists to distinguish between the functions of the two peptides. To circumvent these experimental difficulties, we employed the application of Ab's directed toward ACTH or α-MSH to block the actions of one or both peptides in vivo. A similar approach was previously used successfully to distinguish between the different roles of corticotropin-releasing factor (CRF) and urocortin, both ligands of the CRF2 receptor (33). In addition to the particular feasibility of an Ab approach under the given circumstances, the administration of Ab's possesses further advantages compared with other experimental methods, particularly with respect to bioavailability; i.e., Ab's can exert their actions without needing to access the intracellular compartment, and they are very stable in biological tissues (45). Furthermore, it has been shown in rats that, after administration into the lateral ventricle, Ab's distribute through the whole brain by diffusion within a few hours (12) and that they accumulate in areas where the immunogenic epitope(s) is/are expressed (44). Thus, an application of Ab into one PVN will also cover the surrounding nuclei and the contralateral PVN.

We first experimentally tested whether the PVN and/or surrounding areas are major targets for the anorexigenic actions of icv ACTH and α-MSH; at the same time, the in vivo effectiveness of the Ab's was tested. Both icv ACTH and α-MSH significantly decreased food intake in experimental animals. The finding that local PVN application of the respective Ab abolishes the effects of both melanocortins strongly indicates that alterations in food intake are mediated via either the PVN or sites in close proximity, i.e., the anterior hypothalamic area (AHA). This is supported by previous experiments, in which the PVN was shown to be a major target for NDP-MSH, a stable analog of α-MSH. In those experiments, within the hypothalamus, the PVN, DMN, and medial preoptic area (MPO) were particularly responsive to NDP-MSH, whereas the ARC, LHA, and AHA were less responsive (20). The particular importance of the PVN for melanocortin action is further supported by a study employing local applications of

Table 1. *Effects of ACTH Ab on day- and nighttime food intake in male Wistar rats*

Daytime and Nighttime Food Intakes							
Daytime							
Food intake, g	1 day	2 days	3 days	4 days	5 days	6 days	7 days
IgG	4.9 ± 0.4	5.7 ± 0.3	6.6 ± 0.3	5.9 ± 0.3	6.3 ± 0.4	5.4 ± 0.4	6.0 ± 0.4
ACTH Ab	6.3 ± 0.4*	6.8 ± 0.3*	7.4 ± 0.3*	7.7 ± 0.5*	7.7 ± 0.4*	7.8 ± 0.2*	7.5 ± 0.3*
Nighttime							
Food intake, g	1 day	2 days	3 days	4 days	5 days	6 days	7 days
IgG	14.9 ± 0.6	17.5 ± 0.5	16.6 ± 0.8	17.2 ± 0.7	18.0 ± 0.3	18.4 ± 0.4	19.1 ± 0.7
ACTH Ab	13.7 ± 1.1	16.8 ± 0.4	16.2 ± 0.7	16.8 ± 0.6	17.1 ± 0.3	17.6 ± 0.6	18.0 ± 0.5

Values are means ± SE. Ab, antibody. *$P < 0.05$ vs. IgG-injected rats ($n = 7$ per group).

both an MC3/4R antagonist and agonist, respectively (16). Furthermore, the functional importance of PVN MC4R signaling was shown by selective disruption of MC4R gene transcription and reactivation of expression in the respective area (3). However, to our knowledge, no studies have been undertaken to examine the physiological importance of endogenous POMC cleavage products.

Physiologically, within the cell, the enzymes involved in the processing of α-MSH to ACTH are mainly localized in peptide-containing dense core secretory vesicles and may be excreted with the content of these vesicles, where they theoretically could process extracellular ACTH derived from microinjection. However, for PC2 it is known that the pH optimum of the enzyme is in the 5–6 range (10, 23, 27, 48), corresponding to the moderately acidic pH of the interior of the secretory vesicles. Furthermore, PC2 is strongly activated by Ca^{2+} (42, 47), with an optimum of 1–2 mM in type I secretory granule of the β-cells and liver Golgi (31). Thus, it is unlikely that PC2 is capable of processing ACTH extracellularly, but cannot be ruled out completely, since in cell culture PC2 is capable of cleaving ACTH in the absence of storage granules (4). Furthermore, the cell surface membrane-bound protein furin (42) might be involved in extracellular processing of ACTH; it is expressed in the hypothalamus (11, 40), i.e., the ARC (19). Furin and the PCs cleave at the same general motif (Lys/Arg)-$(Xaa)_n$-(Lys/Arg) ↓ , where $n = 0$, 2, 4, or 6 and Xaa is usually not Cys (↓ indicates cleavage site) (42), and it was speculated that in HMC-1 cells, which do not express PC2, furin is involved in the generation of α-MSH (2).

In our second experiment, we addressed the question of whether ACTH itself is responsible for the anorexigenic effect observed after central application of this peptide or whether proteolytic cleavage into α-MSH is required. We therefore studied the effect of a local application of α-MSH Ab (2 μg/rat) into the PVN in combination with icv administration of ACTH (2 μg/rat). We hypothesized that ACTH itself, without proteolytic processing, does act at the MC4R, and thus α-MSH Ab would not affect the anorexigenic effects of ACTH icv. As expected, the application of ACTH icv in combination with IgG into the PVN significantly reduced food and water intake as well as body weight gain compared with control animals receiving saline icv and IgG into PVN. The PVN application of α-MSH Ab simultaneously with ACTH icv did not affect ACTH's anorexigenic effects. This ineffectiveness of the Ab clearly is not a consequence of an inadequate experimental setting, e.g., because of an Ab not effective in vivo or applied at a site where ACTH does not exert significant effects or insufficient availability of the Ab at the hypothalamus, etc., since in our first experiment, discussed above, we showed that the α-MSH Ab applied into the PVN completely abolished the anorexigenic effects of α-MSH administered icv. Our observations clearly show that icv ACTH itself was affecting food intake and not its proteolytic cleavage product α-MSH. Consequently and importantly, the PVN application of α-MSH Ab was without effect on food and water intake as well as body weight development when ACTH was administered into the lateral ventricle.

The physiological relevance of endogenous ACTH in appetite regulation is indirectly supported by the finding that PC2 knockout mice, which are unable to process ACTH into α-MSH, exhibit normal weight during their lifespan (14). However, one must take into account the fact that compensatory mechanisms may develop during ontogenesis in these animals and also that, since PC2 is involved in processing of a number of peptides (e.g., insulin, thyrotropic hormone), many other factors might contribute to the animals' phenotype in addition to the lack of α-MSH. The role of PC1 in the regulation of POMC processing on body weight homeostasis was emphasized in a recent review (38); e.g., the expression of PC1 mRNA is regulated by leptin or its downstream targets and it has been shown that, in *ob/ob* mice, which lack functional leptin and therefore activation of the melanocortin system by leptin receptors, PC1 mRNA is differentially expressed in hypothalamic nuclei compared with wild-type animals. The expression is also regulated by energy status, whereas no differences in expression were observed for PC2 in different genetic backgrounds or energy status (32).

Interestingly, in our experimental animals, which were 24-h starved by the time of central nervous application, the application of α-MSH Ab into the PVN was without effect on food and water intake as well as on body weight development, supporting our findings in the fasting model in the third experiment, *Effects of endogenous ACTH and α-MSH in or near the PVN on food intake and body weight in satiated and hungry rats* (see below).

Having demonstrated that icv ACTH directly affects body weight homeostasis without processing into α-MSH, we aimed at a more physiological model to study the potential roles of endogenous ACTH and α-MSH. To accomplish this, we established two animal models by subjecting rats to two different food regimens that rendered them either hungry or satiated (see METHODS for details) at the time of Ab application. In fasted rats, endogenous levels of POMC-derived peptides are low, whereas they are high in satiated rats (35, 37). In both animal models, Ab directed toward ACTH, α-MSH, or both Ab's were administered, and food and water intake as well as body weight development were documented. We hypothesized, that in satiated rats with high ACTH and α-MSH levels the application of either one or both of the Ab's would affect food intake and body weight gain by lowering endogenous peptide levels, thus attenuating MC4R activation. As described in RESULTS, we were able to confirm our hypothesis both in animals with single application of substances and in experiments employing three different treatments in a pseudo-random fashion. Ab's effectively elevated food intake and body weight gain; thus, apparently both ligands must have participated significantly in receptor activation. Unexpectedly, however, there was no additive effect when both Ab's were supplied together. A possible explanation for this finding is that gastric distention or other consequences of food intake might feed back to the CNS to activate mechanisms reducing food intake independently of the melanocortin system and therefore are unaffected by immunological blockade of MCR ligands. For example, it has been shown that Agouti mice, in which CNS melanocortin receptors are completely blocked by ectopic expression of agouti protein, are sensitive to the anorexigenic effects of the gastrointestinal peptide $PYY_{3–36}$ (28). Furthermore, it was demonstrated that xenin, a recently discovered gastrointestinal peptide, regulates food intake independently of the melanocortin system (24). This might counteract the Ab-induced increase of food intake and limit the overall effects of a combined administration of both Ab's. However, from the results of the present experi-

ments, one can only speculate, and there may be other explanations for our observation.

In contrast to the findings in satiated rats, in hungry animals with low levels of endogenous POMC-derived peptides (35, 37), application of either ACTH Ab, α-MSH Ab, or both Ab's was without effect on any variable measured, indicating that in hungry rats no effect can be obtained by immunological blockade of the already low α-MSH and ACTH levels. These experiments clearly demonstrate for the first time that not only α-MSH but also ACTH to a similar extent is involved in the regulation of food intake and body weight homeostasis under physiological conditions, since hungry rats were not food deprived but rather had the normal level of appetite at the beginning of the feeding period.

In the final experiment, *Subchronic blockade of endogenous ACTH in the PVN and areas in close proximity,* we examined the effects of immunological blockade of ACTH in a subchronic application scheme with twice daily PVN injections to test whether blockade of ACTH would remain effective for an extended period of time and also to discriminate between the Ab's effects in different feeding states, i.e., in satiated rats (application in the morning) and in hungry rats (application in the evening). Food and water intake were monitored separately for day- and nighttime intake. Comparable to our short-term experiments with single application of Ab (the third experiment), daytime food intake, i.e., food intake in satiated animals, was increased significantly in ACTH Ab-treated compared with control animals; this effect persisted throughout the observation period. In contrast to this and in line with our findings from the third experiment, food intake in hungry animals was only slightly affected by Ab treatment. However, unlike in experiment 3, one must consider that an unaccounted-for number of circadian factors might also affect the parameters measured and therefore the results from experiment 4 cannot be directly compared with experiment 3. Thus, immunological blockade of ACTH obviously also possesses minor effects in hungry animals with low levels of endogenous POMC peptides, which can only be detected when the treatment and observation period is extended.

In summary, we have for the first time shown that endogenous ACTH in the PVN or structures in close proximity plays a crucial role in the physiological regulation of food intake and body weight homeostasis in rats, thus extending current knowledge on the melanocortin system by identifying ACTH as an important physiological ligand to brain MCR in the network of appetite regulation. The PVN and other hypothalamic nuclei in close proximity appear to be of particular relevance for the actions of endogenous ACTH.

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DISCLOSURES

No conflicts of interested are reported by the author(s).

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