Antibodies against the melanocortin-4 receptor act as inverse agonists in vitro and in vivo

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1Applied Pharmacology, Biozentrum, University of Basel, Basel, Switzerland; and 2Centre National de la Recherche Scientifique UPR 9021, Laboratory of Immunological and Therapeutic Chemistry, Institut de Biologie Moléculaire et Cellulaire, Strasbourg, France

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Peter J-C, Nicholson JR, Heydet D, Lecourt A-C, Hoebeke J, Hofbauer KG. Antibodies against the melanocortin-4 receptor act as inverse agonists in vitro and in vivo. Am J Physiol Regul Integr Comp Physiol 292: R2151–R2158, 2007. First published February 22, 2007; doi:10.1152/ajpregu.00878.2006.—Functionally active antibodies (Abs) against central G-protein-coupled receptors have not yet been reported. We selected the hypothalamic melanocortin-4 receptor (MC4-R) as a target because of its crucial role in the regulation of energy homeostasis. A 15 amino acid sequence of the N-terminal (NT) domain was used as an antigen. This peptide showed functional activity in surface plasmon resonance experiments and in studies on HEK-293 cells overexpressing the human MC4-R (hMC4-R). Rats immunized against the NT peptide produced specific antibodies, which were purified and characterized in vitro. In HEK-293 cells, rat anti-NT Abs showed specific immunofluorescence labeling of hMC4-R. They reduced the production of CAMP under basal conditions and after stimulation with a synthetic MC4-R agonist. Rats immunized against the NT peptide developed a phenotype consistent with MC4-R blockade, that is, increased food intake and body weight, increased liver and fat pad weight, and elevated plasma triglycerides. In a separate experiment in rats, an increase in food intake could be produced after injection of purified Abs into the third ventricle. Similar results were obtained in rats injected with anti-NT Abs raised in rabbits. Our data show for the first time that active immunization of rats results in a phenotype that is consistent with hypothalamic MC4-R blockade. Because of their efficacy as inverse agonists and noncompetitive antagonists, these Abs could be used as well-defined pharmacological tools and may be a starting point for the development of novel therapeutic agents.

MATERIAL AND METHODS

Characterization of antigen. Peptides corresponding to the NT (NT peptide) (KTSLHLWNRSSHGLHG, residues 11–25 of the rat MC4-R), or a control sequence (CO peptide) (CANISREERVEFLSVPG), unrelated to the MC4-R were synthesized (16).

The Biacore 3000 system, sensorchip CM5, surfactant P20, amine coupling kit containing N-hydroxysuccinimide (NHS) and N-ethyl-
N’-dimethylaminopropyl-carbodiimide (EDC) were purchased from Biacore (Uppsala, Sweden). Agouti-related protein (AgRP) (83–132) was obtained from Phoenix Pharmaceuticals (Belmont, CA). All biosensor assays were performed with HEPES-buffered saline (HBS) as running buffer (10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, 0.02% surfactant P20, pH 7.4). The analytes AgRP (83–132), NT peptide or CO peptide were diluted in HBS. The sensorchip surface was regenerated after each experiment by injection of 50 mM HCl for 1 min.

NT, CO peptides, and AgRP (83–132) were immobilized on a sensor chip via their free primary amine, using the carboxylated dextran surface activated with 50 μl of a mixture of 0.2 M EDC and 0.05 M NHS. Immobilization of the peptides via their N-terminal part was performed by injecting, onto the activated surface, 35 μl of a peptide solution (10 μg/ml in formate buffer, pH 4.3), which gave a signal of ~40 resonance units (RU) followed by 20 μl of ethanolamine hydrochloride, pH 8.5, to saturate the free activated sites of the protein.

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matrix. Immobilization of AgRP (83–132) was performed as described above with a final signal of 120 RU.

**Animals.** Male Sprague-Dawley rats, 6 wk old at the beginning of the experiments (initial weight ~200 g), were obtained from Charles River (Charles River Laboratories, L’Arbresle, France) and kept at a 12:12-h light-dark cycle (lights on from 6:00 AM to 6:00 PM) in a room with a constant temperature of 22°C and humidity of 50%. Rats were housed individually 1 wk before the beginning of an experiment and were given free access to standard laboratory chow (Nafag 3432, 3.0 kcal/g, 61.6% of total calories from carbohydrate, 24.8% from protein and 13.6% from fat) and tap water. All experiments were performed in accordance with the Swiss regulations for animal experimentation.

**Active immunization in rats.** Rats were divided into three groups: an untreated control group (Sham, n = 5), and two groups that were immunized with the NT (n = 5) or the CO peptide (n = 5). Peptides were applied subcutaneously, behind the neck (25 μg peptide/rat in 0.2 ml Complete Freund’s Adjuvant for the first injection). Thirty and sixty days later, rats received two booster injections of peptides in Incomplete Freund’s Adjuvant. To estimate the efficacy of the immunization procedure, blood was collected before each injection by tail bleeding under isoflurane anesthesia, and the presence of anti-peptide Abs was assessed by ELISA test. Body weight and food intake were recorded three times per week at 9:00 AM.

At the end of the experiments rats were killed by decapitation at 9:00 AM under isoflurane anesthesia. Blood was collected in EDTA-treated tubes after decapitation of the animals, and liver and fat pads (epididymal and retroperitoneal) were removed and weighed. Blood was centrifuged at 2000 g for 20 min at 4°C. Plasma insulin was measured using Insulin Rat Ultrasonic ELISA (DRG Instruments, Marburg, Germany). Plasma glucose was measured using glucose RTU kit and triglycerides by means of a TG PAP 150 kit (both from Biome´rieux, Marcy l’Etoile, France).

**Active immunization in rabbits.** Rabbıts (female New Zealand White rabbits, initial weight ~2.5 kg) (Grimaud, Rousays, France) were immunized using the same procedure as in rats (100 μg of NT peptide in 1 ml of complete Freund’s adjuvant) but boosted only once 3 wk after the first immunization. Total blood was collected 6 wk after immunization under isoflurane anesthesia.

**Determination of the immune response by direct ELISA.** NT and CO peptides (2 μM) were adsorbed with carbonate buffer (Na2CO3, 15 mM, NaHCO3, 35 mM, pH 9.6), on 96-well microtiter plates (BD Biosciences, San Jose, CA), 50 μl/well, by incubating for 1 h at 37°C or overnight at 4°C. Plates were saturated with PBS (NaHPO4, 10 mM, NaCl 150 mM, KCl 27 mM, pH 7.4) supplemented with 3% dried milk (Bio-Rad, Hercules, CA) and 0.05% Tween 20 (Fluka, Buchs, Switzerland) (PBS-Tween milk) for 1 h at 37°C. Serial dilutions of rat sera were added to the plates and incubated for 1 h at 37°C. Plates were then washed with PBS containing 0.05% Tween 20 (PBS-T) and incubated with goat anti-rat IgG horseradish peroxidase (Jackson Immunoresearch Laboratories, San Diego, CA), diluted 1/5,000, for 1 h at 37°C. After the plates were washed with PBS-T and PBS, enzymatic reactions were carried out at room temperature by adding 3,3’5,5’-tetramethylbenzidine in the presence of 0.04% H2O2. Reactions were stopped after 15 min by the addition of HCl (1 N). Optical density was measured at 450 nm by using a microplate reader Victor2 Wallac (Perkin Elmer, Fremont, CA).

**Purification of Abs.** The Abs from anti-NT rat or rabbit sera or anti-CO rat sera were affinity-purified on NT or CO peptide coupled by their N-terminus end to activated CNBr-Sepharose 4B column (Amersham Biosciences, Uppswala, Sweden), according to manufacturer’s instructions. Sera diluted 10 times in PBS were loaded on the column at 4°C. The Abs were eluted with 0.2 M glycine pH 2.7, collected in tubes containing 1 M Tris buffer pH 8, subsequently dialyzed against PBS overnight at 4°C and finally stored at ~20°C.

**Cell culture.** HEK-293 cells expressing hMC4-R were cultured in DMEM (Sigma, St. Louis, MO) containing 10% fetal calf serum (Bioconcept, Allschwill, Switzerland), 1% penicillin/streptomycin (GIBCO, Grand Island, NY) and G418 at 600 μg/ml (Sigma) in a humidified atmosphere containing 5% CO2 at 37°C.

**Immunocytofluorescence experiments.** HEK-293 cells expressing hMC4-R or hMC3-R were fixed for 5 min with 2% paraformaldehyde in PBS. Slides were saturated with PBS supplemented with 5% non-fat dried milk. Anti-NT or anti-CO peptide Abs (50 μg/ml) were applied on cells for 1 h at room temperature. After three washes with PBS, goat anti-rat Alexa conjugated (1/500, Molecular Probes, Junction City, OR) was allowed to react with the fixed primary antibody for 1 h at room temperature. 4’,6-diamidino-2-phenylindole, dihydrochloride (1 μg/ml, Molecular Probes) was used for nuclear staining. The same magnification (×400) and exposure time (500 ms) were used for each slide.

**cAMP assays.** Cells were transferred to 96-well culture plates 12 h before treatment, then washed for 3 h with culture medium (DMEM; Sigma) and incubated for 30 min in PBS supplemented with 0.1% BSA and IBMX (Sigma). Cells were treated with increasing concentrations of hAgRP (83–132) (0.2 nM–2 μM) preincubated with peptides (10 μM) or buffer. Cells were then treated with serial dilutions of purified rat or rabbit Abs for 30 min or preincubated with 25 μM rat Abs or 0.1 μM for rabbit Abs (maximum possible concentrations) for 30 min and then treated with serial dilutions of Merck Compound 1 (23) (10^-9 to 10^-5 M) for 15 min. Merck Compound 1 was selected for these in vitro experiments because it is routinely used in our in vivo experiments (17) due to its high selectivity for the MC4-R. Subsequently, cells were lysed with Biotrak cAMP lysis buffer, and cAMP was measured using the Biotrak cAMP enzyme immunoassay system (Amersham Bioscience, Uppsala, Sweden), according to the manufacturer’s instructions. Protein concentration was determined using the BCA kit (Pierce, Rockford, IL). The concentration of cAMP was expressed in femtomoles cAMP per microgram of protein.

**Intracerebroventricular cannulation.** Male Sprague-Dawley rats (275 to 325 g) were anesthetized with isoflurane in medicinal oxygen (4% for induction and 2% for maintenance of anesthesia). A stainless-steel cannula (26 gauge, 10 mm long) was implanted into the third cerebral ventricle using the following coordinates, relative to the bregma: ~2.5 mm anteroposterior, 0 mm lateral to the midline, and ~7.5 mm ventral to the surface of the skull. The guide cannula was secured in place with three stainless-steel screws and dental acrylic, and a stylet was inserted to seal the cannula until use. Temgesic (Essex Chemie AG, Lucerne, Switzerland) (0.03 mg/kg) was given subcutaneously for 2 days postsurgery. Seven days after recovery from surgery, accuracy of the cannula placement in the third ventricle was tested by measuring the diposogenic response (immediate drinking of at least 5 ml water in 15 min) to an intracerebroventricular injection of 20 pmol of ANG II in 5-μl injection volume.

**Intracerebroventricular injections.** Purified rat or rabbit Abs were slowly (1 min) injected intracerebroventriculatly at 9:00 AM at a dose of 0.1 μg (rabbit and rat Abs) or 1 μg (rat Abs) in a volume of 5 μl using a Hamilton syringe. These doses were selected on the basis of the results of comparative in vitro studies. Following the injection of rat Abs, food intake was continuously recorded during the following 3 days using an automatic food intake apparatus (TSE Systems, Bad Homburg, Germany) at 1-h intervals for 3 days. After the injection of rabbit Abs, food intake was measured during the following 3 days at 9:00 AM and 5:00 PM.

**Intravenous injections.** In a separate experiment, purified rabbit anti-NT Abs, rabbit anti-CO Abs or saline was injected intravenously in the tail vein at 9:00 AM to rats under mild isoflurane anesthesia (n = 8/group) at a dose of 500 μg/kg. Following the injection of Abs, food intake was continuously recorded during the following 3 days using an automatic food intake apparatus (TSE Systems, Bad Homburg, Germany) at 1-h intervals for 3 days.

**Data analysis.** All data are expressed as means ± SD or SE, as indicated. Data were analyzed by two-way ANOVA repeated mea-
sures with Bonferroni post hoc test or by Student’s t-test using GraphPad Prism 4 software. For the cAMP concentration-response experiments, the best fitting curves were compared for their minimum, maximum, and EC50 using F-test.

RESULTS

Characterization of antigen. The physicochemical interaction between NT peptide and AgRP (83–132) was assessed by surface plasmon resonance. When AgRP (83–132) was added to the running buffer, it interacted with immobilized NT peptide with an affinity in the millimolar range. When NT peptide was used in solution and AgRP (83–132) was immobilized, a stronger interaction was measured (KD = 1.77 × 10⁻⁴ M).

The pharmacological effect of the interaction was studied on HEK-293 cells transfected with hMC4-R. When these cells were treated with increasing concentrations of AgRP (83–132) (0.2 nM–2 μM), cAMP formation decreased in a concentration-dependent manner by up to 80%. The NT peptide, at a concentration of 10 μM, which was the highest possible concentration in terms of solubility and tolerability of the cells, displaced the curves slightly but significantly to the right (P = 0.02, F-test). CO peptide had no effect on the inverse agonist activity of AgRP (83–132).

Generation of Abs. All rats immunized with NT peptide developed anti-NT Abs (Fig. 1A). No cross reaction with NT peptide was observed in the rats immunized with CO peptide (Fig. 1B). Conversely, all rats immunized with CO peptide developed anti-CO antibodies (Fig. 1C), which did not cross react with the NT peptide (Fig. 1D).

In vitro characterization of Abs. Figure 2A shows specific membrane labeling of hMC4-R expressing HEK-293 cells when using the NT Abs. No labeling was observed on cells expressing the hMC3-R (Fig. 2B) or on untransfected cells (Fig. 2C). When HEK-293 cells expressing hMC4-R were treated with anti-CO Abs, no staining could be observed (Fig. 2D).

To detect inverse agonist activity of the anti-NT Abs, HEK-293 cells were treated with purified anti-NT Abs. When HEK-293 cells transfected with the MC4-R were exposed to increasing concentrations of anti-NT Abs (2 nM–0.2 μM), cAMP formation decreased in a concentration-dependent manner by up to 40% (Fig. 3A). No decrease in cAMP was measured in response to increasing concentrations of purified anti-CO Abs (Fig. 3A). The presence of anti-NT Abs slightly but significantly (P < 0.001, F-test) shifted the concentration-response curve of Merck Compound 1 to the right and reduced the maximum effect (Fig. 3B).

When HEK-293 cells transfected with the hMC4-R were treated with increasing concentrations of rabbit anti-NT Abs (1 pM–0.1 μM), no changes in cAMP formation were observed (Fig. 3C). In the presence of rabbit anti-NT Abs, the maximum effect of Merck Compound 1 was significantly (P < 0.001, F-test) reduced (Fig. 3D).

Phenotype after active immunization. The rats immunized with the NT peptide (NT group) showed a gradually stronger increase in body weight compared with the CO and sham groups (Fig. 4). The difference between the body weight of rats from the NT group vs. the control groups became significant by
day 35 postimmunization, that is, 5 days after the first boost injection.

At the end of the experiments, the body weight of rats in the NT group was significantly higher than that of rats in the CO group (Table 1). The cumulative food intake during the whole experiment was increased in the NT group compared with the control group (Table 1). At the end of the experiments (day 85), 24-h food intake was increased (Table 1).

Plasma glucose was similar in the NT and CO peptide group. Plasma levels of insulin tended to be higher in the NT group. Plasma triglyceride levels were significantly higher in the NT group (Table 2).

Liver weight and fat pad weight were significantly higher in the NT than in the CO group (Table 2).

**Phenotype after passive immunization.** Two doses (0.1 and \(1 \mu g\)) of purified rat anti-NT and anti-CO Abs were injected into the third ventricle of rats. The lower dose, 0.1 \(\mu g\), of Abs did not induce a change in food intake (Fig. 5A). Rats that received the higher dose, 1 \(\mu g\), of anti-NT Abs ingested 28% more food in 24 h than rats that received anti-CO Abs or saline (Fig. 5B).

When 0.1 \(\mu g\) of purified rabbit anti-NT Abs was injected into the third ventricle of rats, a significant increase in food intake was measured at 24, 48, and 72 h after administration (Fig. 5B). The magnitude of this increase was comparable to the orexigenic effect of the rat anti-NT Abs (Fig. 6, right).

When purified rabbit anti-NT Abs were injected intravenously to rats at a dose of 500 \(\mu g/kg\), no increase in food intake was measured during the following 24 h (data not shown).

Food intake at 48 and 72 h was also not affected (data not shown).

**DISCUSSION**

The goal of the present experiments was to generate pharmacologically active Abs against a central GPCR in rats. The selection of a functionally relevant extracellular sequence of the receptor as an antigen was a prerequisite for successful immunization. From various studies on spontaneous or targeted mutations of the MC4-R (26–28), it seems that an intact N-terminal domain of the MC4-R is essential for the constitutive activity of this receptor (4, 25). Hence, a short peptide sequence from the N-terminus was synthesized, and its functional properties were assessed in surface plasmon resonance experiments. Despite its short length, weak but significant interactions between the NT peptide and the endogenous inverse agonist, AgRP (83–132), could be demonstrated. The biological significance of these molecular interactions was confirmed by the observation that the NT peptide blocked the AgRP (83–132)-induced decrease in cAMP in HEK-293 cells expressing the hMC4-R.

These findings suggested that the peptide sequence chosen for immunization contained the functionally important amino acids. However, the ultimate goal of the immunization was to achieve Abs against the corresponding sequence of the intact receptor. For this purpose, the NT sequence was injected with complete Freund’s adjuvant but without coupling it to a carrier molecule. As shown in previous experiments, this procedure...
resulted in functionally active Abs against GPCRs (11, 13, 20). Only two booster injections were given. More injections would probably increase the titer of Abs against the free peptide but might reduce the number of Abs with affinity for the corresponding sequence in the native receptor.

The properties of the anti-NT Abs were evaluated in a series of in vitro experiments after purification with immunoadsorption chromatography. By using immunocytochemistry in HEK-293 cells expressing hMC3-R or hMC4-R, specific binding to hMC4-Rs could be demonstrated. Although this result was not unexpected because the N-terminal sequences of MC4-R and MC3-R receptor subtypes show a low degree of homology (10), it clearly demonstrated the subtype selectivity of the NT-Abs. The sequences of the extracellular domains are generally less well conserved than those of the transmembrane domains and are therefore suitable targets for a subtype-selective pharmacological approach (14).

In experiments on HEK-293 cells expressing hMC4-R the anti-NT Abs reduced the basal production of cAMP in a concentration-dependent manner by up to 40%. These effects suggest that the Abs reduce the constitutive activity of the receptor, which is an important component of its physiological function. Mutations in the N-terminal region in humans are associated with severe obesity (25), and comparable effects can be achieved experimentally with overexpression of the endogenous inverse agonist, AgRP (19). Compared with AgRP (83–132), which reduced basal cAMP production by up to 80%, anti-NT Abs showed a lower efficacy.

The Abs antagonized the effects of the selective MC4-R agonist, Merck Compound 1, in HEK-293 cells. This low-molecular-weight compound was originally described by Sebhat et al. (23) as a specific MC4-R agonist. We have used this compound in in vivo studies (17) because of its high selectivity for the MC4-R. When Merck Compound 1 was

![Fig. 3. Intracellular cAMP production in HEK-293 cells transfected with hMC4-R. A: concentration-response curve obtained with purified rat anti-NT Abs (■) or with purified rat anti-CO Abs (●). The concentration-dependent decrease in the intracellular cAMP content suggests an inverse agonist effect of anti-NT Abs. B: concentration-response curve obtained with Merck compound 1 in the presence of 25 µM of rat anti-NT Abs (■), rat anti-CO Abs (gray diamonds) or PBS (●). The rightward shift of the curve combined with reduction of maximum efficacy suggests that rat anti-NT Abs act as noncompetitive antagonists. C: concentration-response curve obtained with purified rabbit anti-NT peptide Abs (■). The rabbit anti-NT Abs had no effect on basal cAMP production. D: concentration-response curve obtained with Merck compound 1 in the presence of anti-NT Abs (■) or PBS (●). The reduced maximum efficacy in the presence of anti-NT Abs suggests that they act as noncompetitive antagonists.](https://www.ajpregu.org/)

![Fig. 4. Evolution of body weight in sham (●, n = 5), NT (■, n = 5), and CO (▲, n = 5) rats during the period of active immunization. Body weight of the NT peptide group is significantly higher compared with the two other groups (P < 0.05, two-way ANOVA). Data are presented as means ± SE.](https://www.ajpregu.org/)

Table 1. Phenotype induced by immunization of rats against the NT peptide (NT group) or CO peptide (CO group)

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<th>NT Group</th>
<th>CO Group</th>
<th>Significance</th>
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<tr>
<td>Body weight, g</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Day 2</td>
<td>229±6</td>
<td>224±6</td>
<td>NS</td>
</tr>
<tr>
<td>Day 85</td>
<td>542±13</td>
<td>492±12</td>
<td>*</td>
</tr>
<tr>
<td>Food intake, g/24 h</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 2</td>
<td>24.8±1.1</td>
<td>22.4±2.4</td>
<td>NS</td>
</tr>
<tr>
<td>Day 85</td>
<td>30.4±0.7</td>
<td>28.0±0.7</td>
<td>*</td>
</tr>
<tr>
<td>Total cumulative</td>
<td>1041.0±26.4</td>
<td>898.2±46.4</td>
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Data are presented as means ± SE. *P < 0.05 (Student t-test). NS, not significant; NT, N terminal; CO, control sequence.
Furthermore, it has been reported that Abs can be dem-
as leptin or insulin appear to have access to the hypothalamus
Under physiological conditions, large molecules such
that in this area the blood-brain barrier is either absent or less
action. In the hypothalamus, the blood-brain barrier is a less
penetration of the Abs into the brain, that is, their passage
across the blood-brain barrier, is a prerequisite. Although in the
present studies, we have not demonstrated the presence of Abs
in the brain, it is likely that they reached their central site of
function. In the hypothalamus, it is likely that the Abs were able to reduce MC4-R activity during basal and stimulated conditions.
The MC4-R was chosen as a target because of its important
role in the regulation of energy balance. The phenotype observed
12 wk after the first injection of the NT peptide was consistent with central MC4-R blockade. Body weight and food intake were higher than in sham-injected rats or in rats immunized with the CO peptide. Because the ratio of food intake/24 h on body weight does not differ between the NT (0.056 ± 0.002), CO (0.057 ± 0.1), and sham (0.056 ± 0.011) groups, the higher body weight of the NT group could be explained by a decrease in energy expenditure. Plasma insulin tended to be elevated, and plasma triglycerides were significantly increased. Interestingly, a recent study described low plasma triglyceride levels in human carriers of an overactive mutant (V103I) of the MC4-R (3). The increase in triglyceride levels in the NT group in our experiments is therefore compatible with MC4-R blockade. Similar findings have been reported in mice with MC4-R knockout (9) or after central administration of antisense oligonucleotides against the MC4-R (18) and are suggestive of insulin resistance. When the phenotype in the present studies is compared with that observed after chronic treatment of rats with AgRP (83–132), it is evident that AgRP (83–132) produced much more pronounced changes in body weight and food intake (24). This difference in phenotype most probably reflects the difference in the efficacy of NT-Abs and AgRP (83–132) as inverse agonists in vitro. However, limited efficacy of NT-Abs due to low concentrations at their hypothalamic site of action cannot be excluded.
For an effect on a target in the central nervous system, the
penetration of the Abs into the brain, that is, their passage
across the blood-brain barrier, is a prerequisite. Although in the
present studies, we have not demonstrated the presence of Abs
in the brain, it is likely that they reached their central site of
action. In the hypothalamus, the blood-brain barrier is a less
important obstacle than in other brain regions because it seems
that in this area the blood-brain barrier is either absent or less
tight. Under physiological conditions, large molecules such
as leptin or insulin appear to have access to the hypothalamus
(5). Furthermore, it has been reported that Abs can be demon-
strated in the brain after peripheral administration (1). In active
immunization experiments, Abs are circulating over periods of several weeks. It may be assumed that under these conditions, a more favorable equilibrium between brain and plasma concentrations is achieved. Once Abs have gained access to the brain, they appear to remain there for a prolonged period of time (1).
To find out whether the effects of anti-NT Abs on food intake can be reproduced by passive immunization, purified rat Abs were administered intracerebroventricularly into the third ventricle. When given at the beginning of the light phase, the anti-NT Abs increased 24 h food intake by ~25%. This acute effect is not too different from that obtained with SHU 9119, a peptidic MC3/MC4-R antagonist (Ref. 18 and unpublished data). The main effects on food intake were seen during the dark phase. Slow tissue penetration of Abs may have precluded an earlier onset of action.
Because the amounts of Abs that are obtained in rats are limited, no further experiments with rat Abs could be performed, and rabbits were immunized to produce larger quantities of Abs. Another objective of the studies in rabbits was to

Table 2. Plasma parameters and organ weights of rats immunized against the NT peptide (NT group) or CO peptide (CO group)

<table>
<thead>
<tr>
<th>Plasma concentrations</th>
<th>NT Group</th>
<th>CO Group</th>
<th>Significance</th>
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<tr>
<td>Glucose, mmol/l</td>
<td>6.7 ± 0.3</td>
<td>6.7 ± 0.7</td>
<td>NS</td>
</tr>
<tr>
<td>Insulin, pmol/l</td>
<td>25.1 ± 3.6</td>
<td>18.3 ± 4.7</td>
<td>NS</td>
</tr>
<tr>
<td>Triglycerides, mmol/l</td>
<td>2.4 ± 0.1</td>
<td>1.4 ± 0.4</td>
<td>†</td>
</tr>
<tr>
<td>Liver weight, g</td>
<td>20.5 ± 1.0</td>
<td>16.6 ± 0.9</td>
<td>*</td>
</tr>
<tr>
<td>Fat pads weight, g</td>
<td>23.1 ± 2.6</td>
<td>14.9 ± 0.9</td>
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Data are presented as means ± SE. *P < 0.05, †P < 0.01 (Student’s t-test).

applied to HEK-293 cells expressing hMC4-Rs in the presence of NT-Abs, its concentration-response curve was shifted to the right. Moreover, its maximum efficacy was reduced, suggesting a noncompetitive mode of action, a pattern that has been described for other inverse agonists at GPCRs (21). Because of the combination of inverse agonistic and antagonistic properties, these Abs should be able to reduce MC4-R activity during a noncompetitive mode of action, a pattern that has been described for other inverse agonists at GPCRs (21). Because of the amounts of Abs that are obtained in rats are limited, no further experiments with rat Abs could be performed, and rabbits were immunized to produce larger quantities of Abs. Another objective of the studies in rabbits was to

![Fig. 5](http://ajpregu.physiology.org) Twenty-four-hour food intake in rats that received an intracerebroventricular injection of either 0.1 µg (A) or 1 µg (B) of rat anti-NT Abs (n = 5), anti-CO Abs (n = 5), or saline (n = 5). The injection of 1 µg anti-NT Abs induced a significant increase in food intake. Data are presented as means ± SE. *P < 0.05, **P < 0.01 (NT vs. CO), and #P < 0.05 (NT vs. saline), repeated-measures two-way ANOVA with Bonferroni post hoc test.
find out whether anti-NT Abs could be generated in another species. Rabbits showed an immune response to NT peptide, but the Abs had a different pharmacological profile. They showed no inverse agonistic activity but were still effective as noncompetitive antagonists in vitro with a higher potency than rat Abs. Upon intracerebroventricular administration, the rabbit anti-NT Abs induced a rise in food intake, which was qualitatively and quantitatively similar to that observed with rat Abs. In both cases, the Abs had a long duration of action, and the difference in food intake was still present after 72 h.

Because sufficient amounts of Abs were obtained from immunized rabbits, intravenous injections could be performed. These experiments were done to rule out the possibility that the biological effects observed after intracerebroventricular administration were due to blockade of peripheral MC4-Rs after leakage of Abs into the circulation. No effects on food intake were seen after a single intravenous administration of rabbit Abs in a dose approximately 100-fold higher than that given intracerebroventricularly. This excludes the contribution of peripheral MC4-R but raises the question of whether peripherally administered Abs can reach a central site of action. The absence of a central effect is consistent with the findings of Banks et al. (1) who observed that after intravenous administration, peak concentrations of Abs in brain tissue are only 0.11% of those in plasma. Further experiments with higher doses of anti-NT Abs and repeated administration are needed to study their brain penetration in more detail.

The present studies demonstrate for the first time that rats immunized with an extracellular sequence of a GPCR located in the brain develop a phenotype consistent with chronic receptor blockade. This experimental approach is not only useful to establish a disease model for receptor dysfunction but also provides Abs which can be used as well-defined pharmacological tools. With the appropriate modifications, such Abs may have the potential to become therapeutic agents.

ACKNOWLEDGMENTS

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