European Journal of Pharmacology 660 (2011) 80–87

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Review

Implication of the melanocortin-3 receptor in the regulation of food intake

Boman G. Irani [a], Zhimin Xiang [a], Hossein N. Yarandi [b], Jerry R. Holder [a], Marcus C. Moore [a], Rayna M. Bauzo [a], Bettina Proneth [a], Amanda M. Shaw [a], William J. Millard [a], James B. Chambers [c], Stephen C. Benoit [c], Deborah J. Clegg [c], Carrie Haskell-Luevano [a,*]

[a] Department of Pharmacodynamics, University of Florida, PO Box 100487, Gainesville, FL-32610, United States
[b] College of Nursing Biostat Unit, University of Florida, PO Box 100187, Gainesville, FL 32610, United States
[c] Department of Psychiatry, University of Cincinnati Medical Center, Cincinnati, OH 45267, United States

ARTICLE INFO

Article history:
Received 1 August 2010
Received in revised form 28 September 2010
Accepted 12 October 2010
Available online 1 January 2011

Keywords:
Melanotropin
MC3R
MC4R
Knockout mice
Obesity
Food intake
Feeding
Energy homeostasis
AGRP
SHU9119

ABSTRACT

The melanocortin system is well recognized to be involved in the regulation of food intake, body weight, and energy homeostasis. To probe the role of the MC_3 in the regulation of food intake, JRH322-18 a mixed MC_3 partial agonist/antagonist and MC_4 agonist tetrapeptide was examined in wild type (WT) and melanocortin 4 receptor (MC_4) knockout mice and shown to reduce food intake in both models. In the wild type mice, 2.0 nmol of JRH322-18 statistically reduced food intake 4 h post icv treatment into satiated nocturnally feeding wild type mice. The same dose in the MC_4KO mice significantly reduced cumulative food intake 24 h post treatment. Conditioned taste aversion as well as activity studies supports that the decreased food intake was not due to visceral illness. Since these studies resulted in loss-of-function results, the SHU9119 and agouti-related protein (AGRP) melanocortin receptor antagonists were administered to wild type as well as the MC_3 and MC_4 knockout mice in anticipation of gain-of-function results. The SHU9119 ligand produced an increase in food intake in the wild type mice as anticipated, however no effect was observed in the MC_3 and MC_4 knockout mice as compared to the saline control. The AGRP ligand however, produced a significant increase in food intake in the wild type as well as the MC_3 and MC_4 knockout mice and it had a prolonged affect for several days. These data support the hypothesis that the MC_3 plays a subtle role in the regulation of food intake, however the mechanism by which this is occurring remains to be determined.

Contents

* Corresponding author. Tel.: +1 352 273 7717; fax: +1 352 273 7723.
E-mail address: Carrie@cop.ufl.edu (C. Haskell-Luevano).

0014-2999/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2010.10.101

1. Introduction

The melanocortin system has been implicated in the regulation of a diverse number of physiological processes including pigmentation, feeding behavior, cardiovascular, and sexual function. The melanocortin 1 receptor (MC_1) is clearly linked to the melanocortin agonist effects on pigmentation and animal coat coloration (Mountjoy et al., 1992). The melanocortin 4 receptor (MC_4) has been demonstrated to be involved in the regulation of food intake (Fan et al., 1997) and therefore has an impact on obesity (Huszar et al., 1997) in rodents and humans. The melanocortin 3 receptor (MC_3) is involved in the regulation of energy and fat homeostasis (Butler et al., 2000; Chen et al., 2000) however, its mechanism of action remains to be identified. The double MC_3/MC_4 knockout mice are extremely obese and hyperphagic (Atalayer et al., 2010) and mounting evidence supports the hypothesis regarding a synergistic role between the MC_3 and MC_4 in the regulation of energy homeostasis and perhaps food intake.

The study reported herein was designed to probe the role of the MC_3 in the regulation of food intake and determine if a melanocortin ligand possessing mixed MC_3/MC_4 agonist/antagonist pharmacology might result in a more desirable anti-obesity therapeutic agent versus a highly selective MC_4 agonist that results in increased blood pressure (Greenfield et al., 2009) and erectile function (Wessells et al., 1998). Table 1 summarizes the melanocortin 3 and 4 receptor pharmacology of the peptides examined in this study as well as the reference compounds α-MSH and NDP-MSH (Haskell-Luevano et al., 2001, 2000; Holder et al., 2002; Proneth et al., 2008; Wilczynski et al., 2004a, 2004b). Fig. 1 illustrates the functional antagonist pharmacology of agouti-related protein (AGRP), SHU9119, and JRH322-18 at the mouse MC3R. The AGRP antagonist does not possess any partial agonist activity at the MC3R receptor whereas both SHU9119 and JRH322-12 possess partial agonist as well as antagonist pharmacological profiles.

2. Materials and methods

2.1. Peptides

The compounds utilized in this study were purchased from commercial sources MTII and SHU9119 (Bachem, Torrance CA), AGRP(86–132) (Peptides International, Louisville KY) or synthesized as previously reported JRH887-9 and JRH322-18 (Holder et al., 2002). Each compound was tested at the multiple doses as indicated. Behavior was monitored continuously for up to 6 h post treatment for adverse toxic or visceral illness. Such behaviors were only observed for the JRH compounds at the 5 nmol doses, and these data have been excluded, and resulted in a lack of food intake, normal activity and/or death during the duration of data collection.

2.2. Animals

All studies performed were conducted in accord with accepted standards of humane animal care and were approved by the Institutional Animal Care and Use Committee at the University of Florida and at the University of Cincinnati. The MC_4 knockout mice on a mixed Bl6/129 background were generously provided by Dr. Dennis Huszar at Millennium Pharmaceuticals (Huszar et al., 1997). The MC_3 knockout mice on a mixed Bl6/129 background were provided by Lex Van Der Ploeg at Merck (Chen et al., 2000). A heterozygous breeding strategy was utilized to generate age/litter mate matched mice. Mice were housed in individual cages in a temperature-controlled room (23–25 °C) and maintained on a 12 h light/dark cycle (lights off at 1800 h). Animals were given free access to normal chow and water at all times.

2.3. Study design

This study utilized a Latin Square non-fasted feeding paradigm where mice were allowed free access to food at all times. Compound or saline control (1 μl in saline) was injected ca 2 h before lights out and food intake (standard chow [Harlan Teklad 8604 diet (24% crude protein, 4% crude fat, and 4.5% maximum for crude fiber with a digestible energy of 3.30 kcal/g)]) was measured at X time points. The bedding was inspected for large intact pellets >0.1 g to decrease the error associated with determining food intake. No significant differences between the wild type littermate mice from either the MC_4RKO or MC_3RKO colonies were observed so the data was combined.

Table 1
Summary of *in vitro* mouse melanocortin receptor pharmacology.

Name	Structure	Functional activity (nM) mMC3R	Binding affinity (nM) mMC3R	Functional activity (nM) mMC4R	Binding affinity (nM) mMC4R
α-MSH	Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2	Agonist 0.80 ± 0.14	ND	Agonist 5.40 ± 0.62	ND
NDP-MSH	Ac-Ser-Tyr-Ser-Nle-Glu-His-DPhe-Arg-Trp-Gly-Lys-Pro-Val-NH_2	Agonist 0.098 ± 0.013	0.52 ± 0.10	Agonist 0.21 ± 0.03	0.91 ± 0.85
MTII	Ac-Nle-c[Asp-His-DPhe-Arg-Trp-Lys]-NH_2	Agonist 0.16 ± 0.03	1.85 ± 0.35	Agonist 0.087 ± 0.008	0.50 ± 0.13
SHU9119	Ac-Nle-c[Asp-His-DNal(2′)-Arg-Trp-Lys]-NH_2	Antagonist[a] 0.32	3.2 ± 1.1	Antagonist 0.040	0.38 ± 0.10
JRH887-9	Ac-His-DPhe-Arg-Trp-NH_2	Agonist 160 ± 9.2	ND	Agonist 17 ± 2.8	ND
JRH322-18	Ac-His-(pI)DPhe-Arg-Trp-NH_2	Antagonist[a] 50 ± 1.1	270 ± 146	Agonist 25 ± 9.8	4.81 ± 4.5
AGRP(86–132)		Antagonist 1.4	2.8 ± 0.4	Antagonist 0.41	3.0 ± 0.3

ND indicates values not reported. The indicated error represents the standard error of the mean.
[a] Indicates that the antagonists also possess partial agonist activity at the mMC3R.

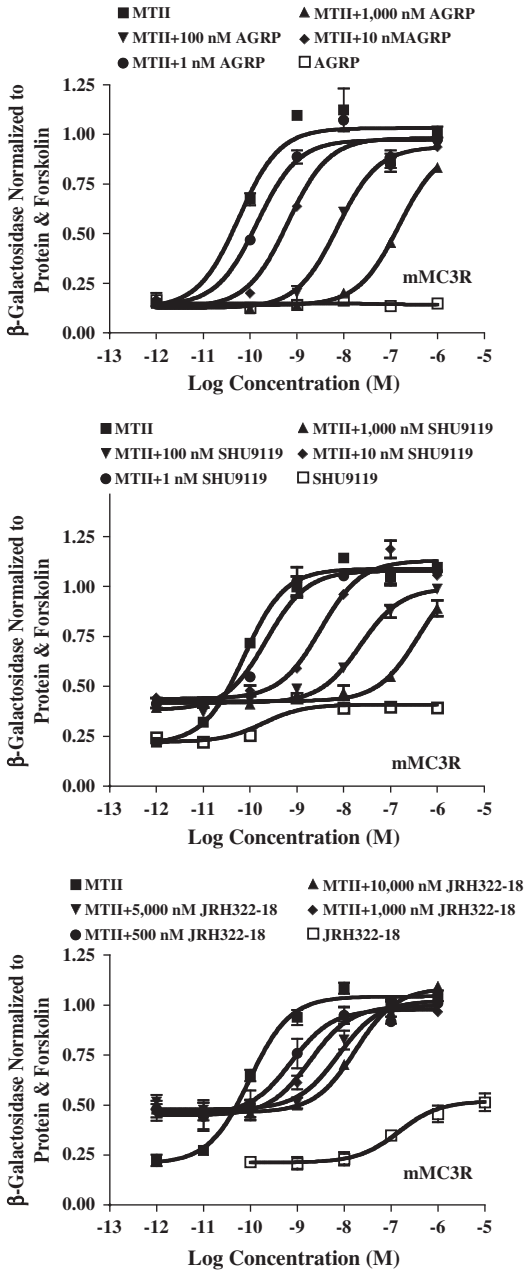

Fig. 1. *In vitro* mouse MC_3 receptor pharmacology of the AGRP, SHU9119, and JRH322-18 peptides examined in this study.

2.4. Cannula surgery and placement validation

Mice were anesthetized with pentobarbital (60 mg/kg) and placed in a stereotaxic apparatus (Cartesian Instruments, Bend, OR). The fur was cut, a midline incision was made, and the exposed area was sterilized. A 25-gauge cannula (Plastics One, Roanoke, VA) was inserted into the lateral cerebral ventricle, coordinates: 1.0 mm lateral and 0.46 mm posterior to bregma and 2.3 mm ventral to the surface of the skull (Franklin and Paxinos, 1997). The cannula was fixed to the skull using a liquid adhesive (Loctite 454, Plastics One, Roanoke, VA) applied to the pedestal of the cannula. After the adhesive dried, dental cement was applied to the exposed skull area and allowed to dry. Subsequently, the skin was sutured and the mice allowed to recover for at least seven days post surgery. Cannula placement was verified by administration of 2 μg human PYY(3–36) (Bachem, Torrance, CA.) as described (Marsh et al., 1999a). For the mice examined in this study, the average grams of cumulative food intake 4 h post treatment for the WT (saline = 0.73 ± 0.06, PYY(3–36) = 1.35 ± 0.08, $p<0.001$), MC_4KO (saline = 0.46 ± 0.11, PYY(3–36) = 1.41 ± 0.13, $p<0.001$), and MC_3KO (saline = 0.76 ± 0.09, PYY(3–36) = 1.60 ± 0.06, $p<0.001$) mice were significantly different. Animals not consuming at least 1 g of food 4 h post injection of PYY(3–36) were excluded from the study.

2.5. c-Fos immunohistochemistry

To study the acute effect of JRH compounds on c-Fos like immunoreactivity in the brain, 3.0 nmol JRH887-9 (n = 3), 3.0 nmol JRH322-18 (n = 3), and 0.9% NaCl (n = 3) were infused into cannulated mice (used previously in feeding studies). Two hours after the infusions, mice were transcardially perfused with 0.9% Phosphate Buffered Saline (PBS) followed by formalin-picric acid mixture (4% paraformaldehyde and 0.4% picric acid in 0.16 M phosphate buffer, pH = 6.9, 37 °C). Brains were rapidly removed and fixed for 16 h and subsequently rinsed in 0.1 M phosphate buffer. Forty-micrometer forebrain slices were cut in the coronal plane on a vibratome to allow visualization of various hypothalamic nuclei including the PVN, DMH, VMH and ARC. Sections were then rinsed (3×, PBS), incubated for 30 min in 0.3% H_2O_2, rinsed (3×, PBS), and incubated 1 h in 0.3% Triton X-100 and 1% normal goat serum in PBS. Sections were then transferred directly to the primary antibody solution consisting 0.005 g/ml polyclonal rabbit antiserum (Santa Cruz Biotechnology, Santa Cruz, CA), which recognizes residues 3–16 of the c-Fos protein. After 36 h of incubation at 4 °C, slices were rinsed (3×, PBS). Slices were then transferred to biotinylated goat anti-rabbit antibody for 1 h, rinsed (3×, PBS), and developed with diaminobenzidine substrate (5 min). Slices were rinsed (3×, PBS) mounted on gelatin coated slides and coverslipped with D.P.X mounting medium (Ft. Washington, PA). The number of c-Fos positive nuclei was quantified in specific hypothalamic nuclei using a Leica DC500 imaging system and the SigmaScan Pro 5.0 Image analysis application (SPSS Science, Chicago, IL). Sections examined for c-Fos-like immunoreactivity (c-FLI) included PVN, DMH, VMH and ARC.

2.6. Conditioned taste aversion

Mice (n = 8) were first habituated to 2 h daily access to water. During this time, two bottles, each containing unflavored water, were placed on each home cage. After 12 days, all mice received two bottles containing 0.1% saccharin instead of water. Immediately following the 2-h exposure, mice received i.p. saline, LiCl (a volume equivalent to 2% of the mouse body weight of a 0.15 M isotonic solution) or drug. On the following day, mice again received 2 h access to two bottles with unflavored tap water. On the final day, a two-bottle choice test was administered in which all mice were allowed access to tap water and the saccharin solution in separate bottles. The relative position of the two solutions was counterbalanced across subjects. The ratio of saccharine intake to total liquid intake during test was calculated and used as the index of aversion.

2.7. Locomotion and other behavioral assays

To assess locomotor activity and gross behavioral changes, the CleverSys Topscan and Sidescan programs (CleverSystem, Inc., Reston, VA) were used. Gross behavioral activity was scored by the software, which provides a detailed matrix of behaviors over time, including velocity, distance traveled, sniffing, location, eating and ambulation. Drugs that elicit visceral illness are known also to reduce overall activity (e.g., belly-lying). We can record and analyze this activity at a resolution of approximately 10 s. Mice (n = 8) were injected with LiCl, saline, or drug under test and the overall index of activity used was an indicator of illness-like effects. Mice were individually placed in specialized cages equipped with a stainless steel running wheel

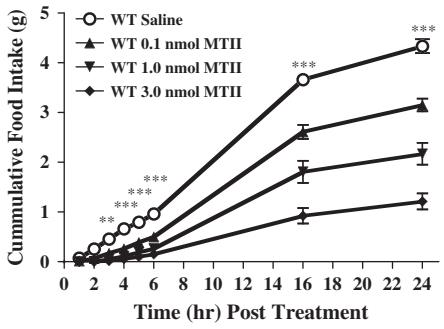

Fig. 2. Effect of MTII on food intake (mean ± S.E.M.) in wild type mice after lateral ventricular administration using a non-fasted paradigm ($p<0.0001$ n = 17/group). The 0.1, 1.0, and 3.0 nmol doses statistically decreased food intake relative to saline starting at t = 4 h, t = 3 h, and t = 3 h, respectively (**$p<0.01$).

(Lafayette Instrument Company, Lafayette, IN). All animals had free access to the running wheel which was monitored with infrared beams connected to a computer which transduced revolutions to distance traveled and velocity. Distance was measured in meters (cumulative) over time across 48 h. All animals had ad lib access to food and water while housed in running wheel cages.

2.8. Statistics

Data is represented as the average of the mean ± S.E.M. For statistical analyses, compound concentration over time was performed using two-way ANOVA followed by Bonferroni post test. Statistical significance is considered if $p<0.05$.

3. Results

3.1. Experiment 1: validation of MTII decreased food intake in satiated wild type mice

To validate our experimental paradigm (non-fasted and lateral ventricle administration compound) and reproduce previous findings (Fan et al., 1997; Marsh et al., 1999a) we tested the MTII melanocortin agonist in wild type mice. These data are consistent with previous reports and are summarized in Fig. 2.

3.2. Experiment 2: effect in wild type and MC_4 mice of a mixed MC_3 antagonist and MC4R agonist tetrapeptide JRH322-18 and the control MC3R and MC4R tetrapeptide agonist JRH887-9

It is well established that the MC_4 is directly involved in the regulation of food intake. However, little is understood about the involvement of the MC_3 in food intake or the effects of a mixed MC_3 antagonist and MC_4 agonist in wild type mice. The JRH887-9 peptide was included as a control peptide due to the structural similarity. The MC_4KO mice were included since it was previously postulated that the melanocortin based feeding effects were only due to the MC_4 and not the MC_3 (Marsh et al., 1999a), even though the MC_3KO mice have been reported to be hypophagic on normal chow (Chen et al., 2000). Thus, for this study, we postulated that we would be able to associate differences in the JRH322-18 mixed receptor pharmacology to that of the MC_3. In the wild type littermate mice, as anticipated based upon the *in vitro* MC_4 agonist pharmacology, JRH887-9 decreased food intake 2 h post treatment ($p<0.05$) at the 2.0 nmol dose (Fig. 3). In the MC_4KO littermate mice, the 2.0 nmol dose significantly decreased food intake ($p<0.001$) 16 h post treatment. Treatment with the mixed MC_3 antagonist/MC_4 agonist JRH322-18

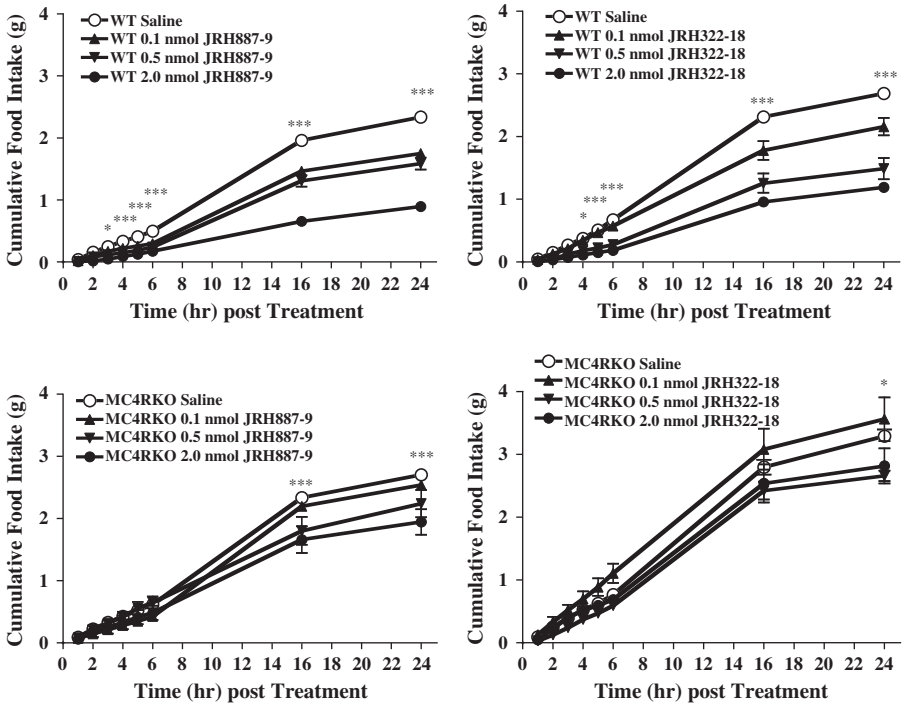

Fig. 3. Effect of the JRH tetrapeptides on food intake (mean ± S.E.M.) in the wild type and MC_4KO littermate mice (n = 10–12 per group wild type and n = 6 per group for the MC_4KO mice). These compounds significantly decreased food intake at the wild type and MC_4KO mice ($p<0.0001$). The tetrapeptide JRH887-9 in the wild type mice, the 0.1, 0.5, and 2.0 nmol doses statistically decreased food intake relative to saline starting at t = 5 h, t = 4 h, and t = 3 h, respectively (*$p<0.05$). In the MC_4KO mice, the 0.5, and 2.0 nmol doses statistically decreased food intake relative to saline starting at t = 16 h (***$p<0.001$). The tetrapeptide JRH322-18 in the wild type mice, the 0.1, 0.5, and 2.0 nmol doses statistically decreased food intake relative to saline starting at t = 16 h, t = 5 h, and t = 4 h, respectively ($p<0.05$). In the MC_4KO mice, the 0.5 and 2.0 nmol doses statistically decreased food intake relative to saline starting at t = 24 h ($p<0.05$).

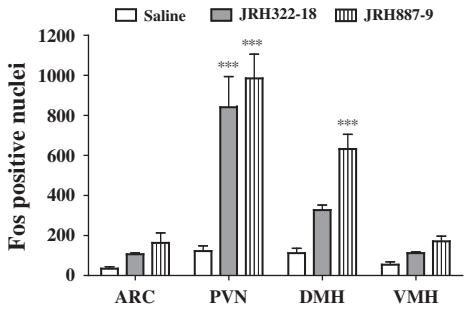

Fig. 4. Effect of JRH treatment on hypothalamic c-Fos-like immunoreactivity in wild type mice 2 h after 3.0 nmol compound administration (n = 3–4 animals per group). Statistical significance is observed for the JRH compounds in the PVN, but only for JRH887-9 in the DMH (***$p<0.0001$). The JRH322-18 compound is an MC_3 antagonist as well as an MC_4 agonist.

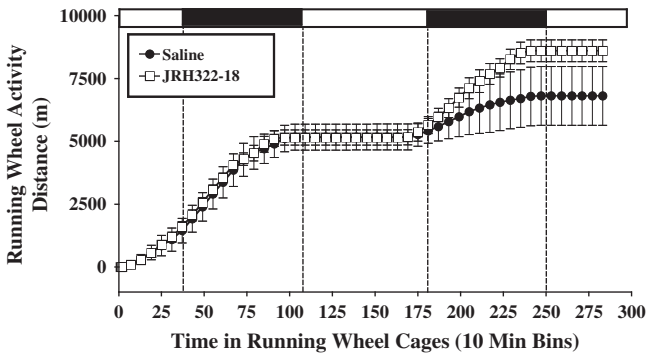

Fig. 6. Locomotor assay to assess visceral illness-like effects and gross behavioral changes of JRH322-18 relative to saline treatment in wild type mice. Mean ± S.E.M. distance traveled (in meters) during 48 h in running wheel cages for mice injected into the 3rd ventricle with 1 μl of either saline (filled circles, n = 5) or JRH322-18 (open box, n = 4). X-axis is time spent in running wheel cages in hours, and Y-axis is distance traveled in meters. Dashed vertical lines show transition points during light–dark cycle, and horizontal bars at top denote light phase (open bar) or dark phase (filled bar). No statistically significant differences resulted and the JRH322-18 treated group demonstrated a slightly increased running wheel activity at the 36 to 48 h post injection time points.

resulted in significantly decreased food intake in the wild type mice at 4 h post treatment ($p<0.05$) and in the MC_4KO mice, 24 h post treatment ($p<0.05$).

The endogenous POMC melanocortin agonist mRNA is synthesized in the arcuate nucleus (ARC) of the hypothalamus and peptide expressing projections innervate the hypothalamic and brain regions that express the MC_3 and MC_4 receptors (Low et al., 1994). Previous studies using α-MSH and c-Fos-like immunoreactivity 2 h post icv administration identified statistically significant differences in the PVN but not the ARC (McMinn et al., 2000). To determine if the JRH compounds could induce c-Fos-like immunoreactivity in similar or different hypothalamic nuclei, we compared c-Fos-like immunoreactivity following treatment and determined immunoreactivity in the following hypothalamic regions: ARC, PVN, DMH, and VMH regions (Fig. 4). Consistent with previous reports, the JRH compounds did not induce significant c-Fos-like immunoreactivity in the ARC (McMinn et al., 2000). We also did not see a significant effect in the VMH. Significant differences in c-Fos-like immunoreactivity were observed for the JRH compounds as compared to saline in the PVN and DMH ($p<0.0001$) when compared to saline control injections. Interestingly however, in the DMH, JRH322-18 (MC_3 partial agonist and antagonist) did not induce a significant c-Fos-like immunoreactivity response.

Reductions in food intake can be due to visceral illness induced by the drug treatment. To test for visceral illness, we performed a conditioned taste aversion experiment. Importantly, the MTII agonist has previously been demonstrated to cause a conditioned taste aversion (Benoit et al., 2003; Thiele et al., 1998). Two hours post treatment, JRH322-18 compound had a modest conditioned taste aversion response that was not evident at the 24-h time point (Fig. 5). It is unknown why this compound resulted in the 2 h conditioned taste aversion response and it is unclear the affect that this may have on the decreased food intake response observed at the indicated time points. To access visceral illness-like effects and gross behavioral changes mice treated with JRH322-18 and saline were monitored using a locomotor assay (Fig. 6). No statistically notable changes in activity upon compound treatment (1.0 nmol) were observed. However the JRH322-18 treated group demonstrated a slightly increased running wheel activity at the 36 to 48 h post injection time points.

3.3. Experiment 3: effect of AGRP(86–132) and SHU9119 MC_3 and MC_4 antagonists in wild type littermate and the MC_3KO and MC_4KO mice

The results of the JRH compounds in the MC_4KO mice were unanticipated. A variety of factors could result in decreased food intake, although the conditioned taste aversion and sickness behavior do not appear to be the cause of the reduction in food intake for the JRH compounds in Experiment 2. It has been previously demonstrated that the endogenous AGRP antagonist stimulates food intake over a prolonged (days) period (Hagan et al., 2000). SHU9119 has also been characterized to increase food intake (Fan et al., 1997), although its duration has not been previously reported. The goal of this experiment was to reproduce the AGRP increased food intake using our experimental paradigm and characterize the feeding pattern in the melanocortin receptor knockout mice. We hypothesized, based upon previous studies, that the wild type and MC_3KO mice that have intact MC_4 receptors would increase their food intake following central AGRP administration. In the MC_4KO mice, with intact MC_3s we also anticipated an increased food intake response to AGRP based upon previous results by Marsh et al. (1999a,b). For this experiment, we examined the hourly cumulative food intake each between 2 to 8 h post compound administration and daily for up to 7 days. Increased food intake and a prolonged cumulative food intake effect of a single treatment of 2.0 nmol AGRP(86–132) resulted in the wild type,

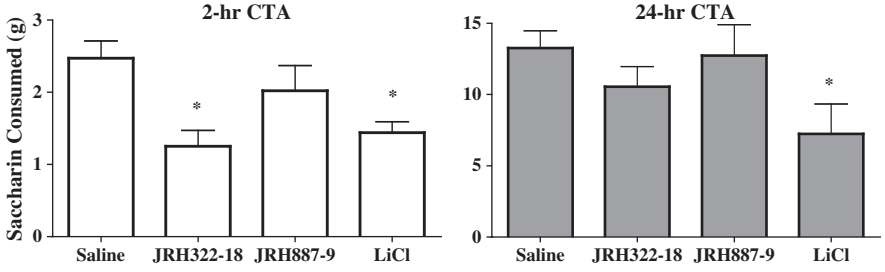

Fig. 5. Effect of JRH treatment (1.0 nmol) in wild type mice in conditioned taste aversion studies (n = 4–5 mice per group). At the 2 h time point, a difference between saline and JRH322-18 (*$p<0.05$) treatment in the amount of saccharin consumed is observed. However, it is not observed at the 24 h time point. LiCl is the lithium chloride control.

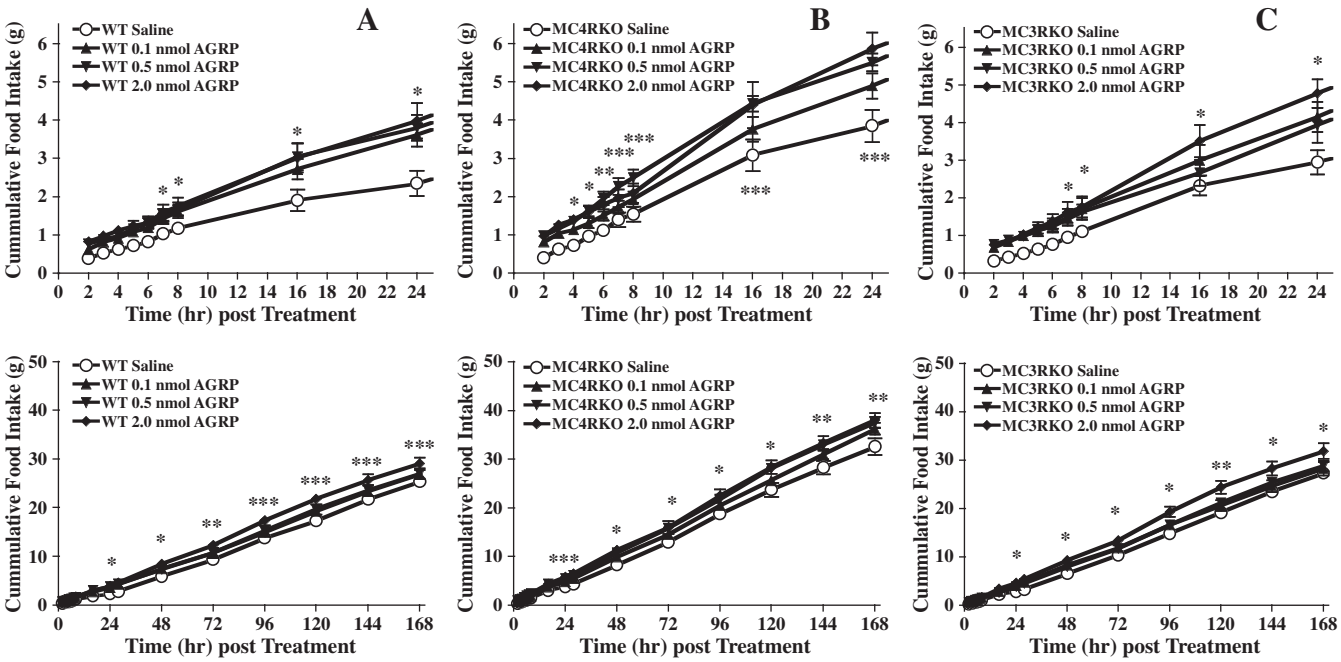

Fig. 7. Effect of AGRP(86–132) on food intake (mean ± S.E.M.) in satiated wild type, MC_4KO, and MC_3KO mice. For all the mice, statistically increased food intake was observed relative to the saline control ($p<0.0001$, n = 8–9 per group). A) In the wild type mice the 2.0 nmol dose statistically increased food intake relative to saline starting at t = 7 h and continued to remain statistically significant for the duration of the experiment (*$p<0.05$). B) In the MC_4KO mice, the 0.5 and 2.0 nmol doses statistically increased food intake relative to saline starting at t = 5 h and t = 4 h, respectively, and continued to remain statistically significant for the duration of the experiment (*$p<0.05$). C) B) In the MC_3KO mice, the 2.0 nmol dose statistically increased food intake relative to saline starting at t = 7 h and continued to remain statistically significant for the duration of the experiment (*$p<0.05$). *$p<0.05$, ** $p<0.01$, and ***$p<0.001$.

MC_3KO and MC_4KO mice (Fig. 7). However, for the SHU9119 antagonist, we only observed significantly increased cumulative food intake in the wild type mouse and only for up to 24 h post treatment at up to 3.0 nmol concentrations (Fig. 8). Comparison of the average 24 h daily intake for mice treated with 2.0 nmol AGRP(87–132) or 3.0 nmol SHU9119 is shown in Fig. 9. Consistent with the previous findings reported by Hagen et al. in the rat (Hagan et al., 2000), AGRP produced a sustained increased food intake for up to six of the seven days measured in the wild type mice. In the MC_3KO mice, which has intact MC_4s, an increase in food intake was observed for days 1, 2, and 4 post treatment. Interestingly, the MC_4KO mice, with intact MC_3s, showed prolonged increased food intake, similar to the wild type mice. SHU9119 treatment in the wild type mice only showed increased food intake one day post treatment. No significant prolonged effect for 3.0 nmol SHU9119 treatment was observed in the wild type, MC_4KO, or MC_3KO mice.

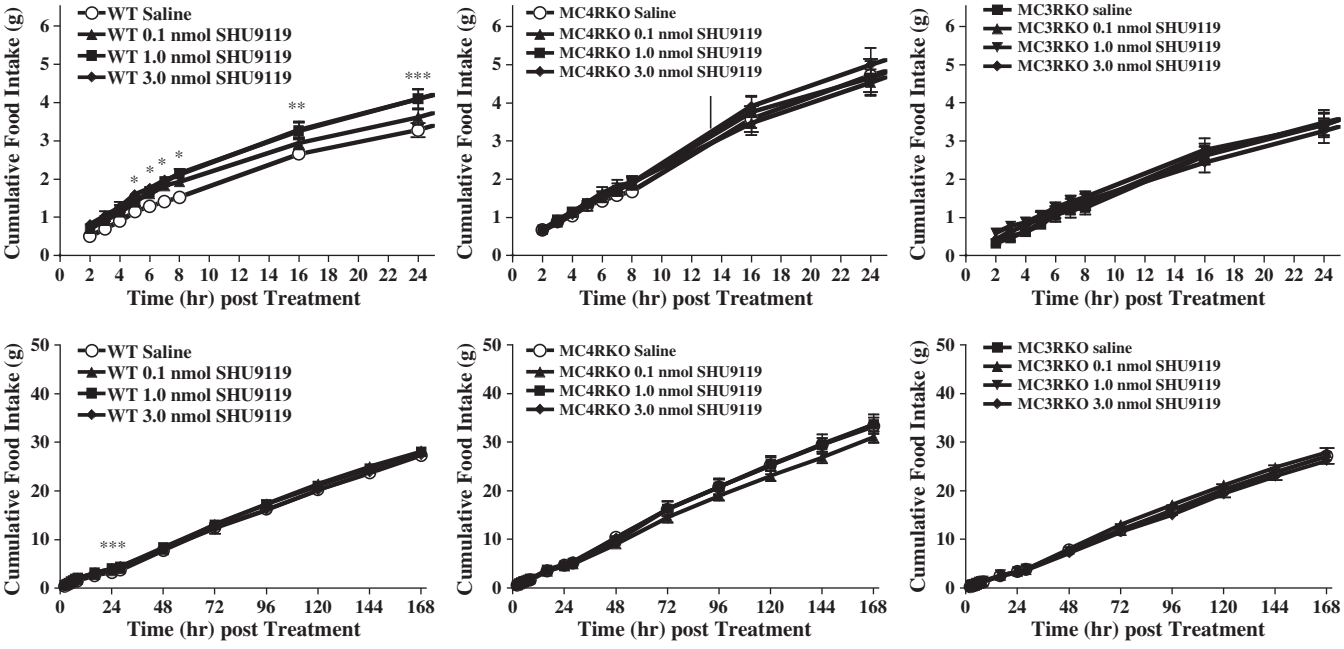

Fig. 8. Effect of SHU9119 on food intake (mean ± S.E.M.) in satiated wild type, MC_4KO, and MC_3KO mice (n = 10–12 per group). In the wild type mice the 1.0 and 3.0 nmol dose statistically increased food intake relative to saline starting at t = 7 h and t = 5 h, respectively, and continued to remain statistically significant up to the 24 h post injection time point (*$p<0.05$). In the MC_4KO and MC_3KO mice, no statistically significant changes in food intake relative to saline were observed.

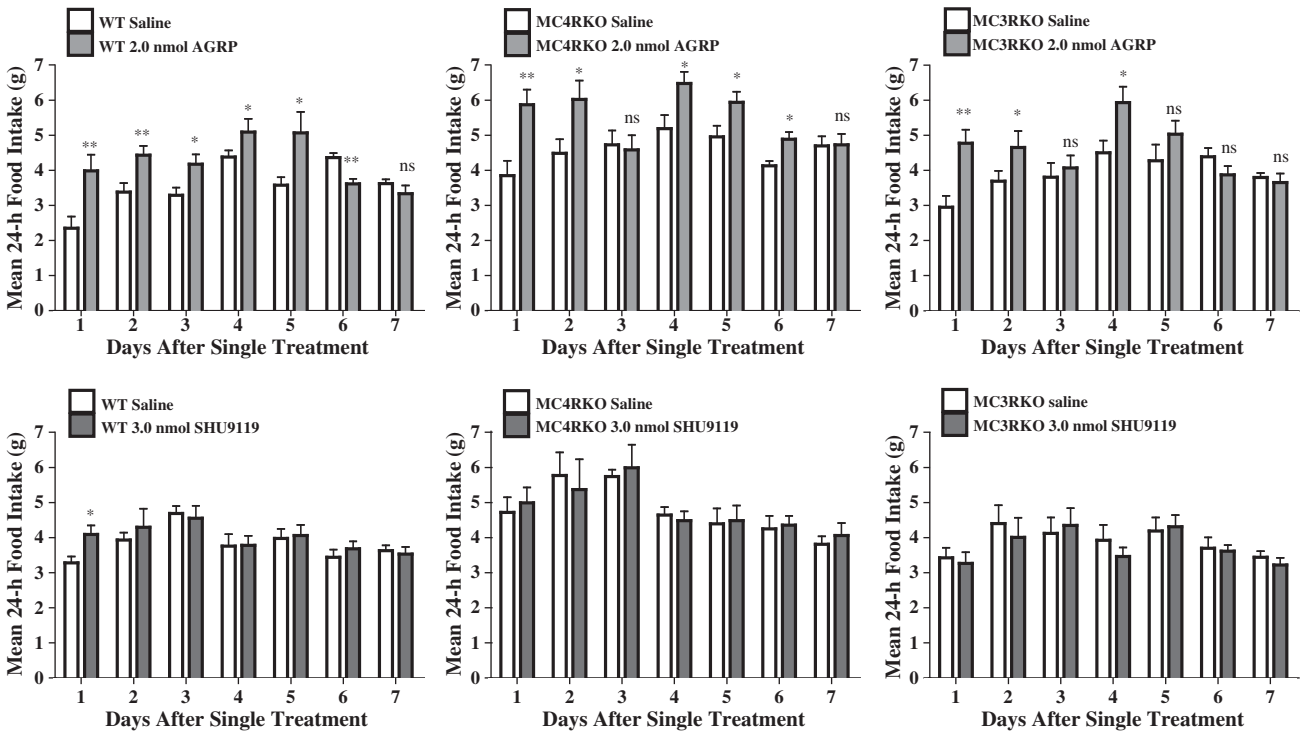

Fig. 9. Shows the average daily food intake (mean ± S.E.M.) per 24 h after a single injection of either AGRP(86–132) or SHU9119 (same groups as in Figs. 7 and 8). In the wild type littermate mice, AGRP(86–132) demonstrated significantly increased average daily food intake versus saline for up to the sixth day of the experiment. *$p<0.05$, **$p<0.01$, and ns = not statistically different.

4. Discussion

The MC_4 has been clearly demonstrated to regulate the control of food intake upon icv administration of agonists (decrease food intake) and antagonists (increase food intake) (Fan et al., 1997). Marsh et al. treated the MC_4KO mouse with increasing doses of the AGRP antagonist and examined food intake 24 h post treatment and identified that AGRP did increase food intake in MC_4KO mice (Marsh et al., 1999b). Chen et al. reported that MC_3KO mice possessed a hypophagic response in a gene-dosage effect compared to both the heterozygous and wild type control mice (Chen et al., 2000). Lee et al. reported that 3 day infusion of D-Trp^8-γ_2-MSH (Grieco et al., 2000), a ca 60-fold mouse MC_3 versus MC_4 selective agonist, stimulates food intake in rats (Lee et al., 2008). Marks et al. (2006) reported that i.p. administration of D-Trp^8-γ_2-MSH at different concentrations and daily versus nocturnal feeding paradigms resulted in both increased and decreased food intake in wild type mice. They also reported that 50 μg i.p. at 4 h post treatment resulted in decreased nocturnal food intake in wild type mice yet the MC_4KO mice increased food consumption upon treatment. While previous reports in the literature suggest that MC_3 D-Trp^8-γ_2-MSH based agonists result in increased food intake under certain feeding experimental paradigms (Lee et al., 2008; Marks et al., 2006), this data is at odds with the highly potent non-selective melanocortin receptor agonist MTII which decreases food intake (Fan et al., 1997) in a variety of feeding paradigms as well as in both rats and mice. Clearly the use of purely selective MC_3 agonists or antagonists (i.e. devoid of any MC4R and MC5R functional activity) would be a valuable tool to discern the role of the MC_3 in the regulation of food intake, however none exist to date. The γ-MSH related analogues are all full agonists at the MC_3 and MC_4 receptors (albeit with differing degrees of receptor selectivity profiles), the SHU9119 is an MC_3 partial agonist and competitive antagonist (Fig. 1) as well as a potent MC_4 antagonist, and AGRP is a potent MC_3 and MC_4 competitive antagonist as well as an inverse agonist at the MC_4 (Haskell-Luevano and Monck, 2001; Nijenhuis et al., 2001) with its potential as an MC_3 inverse agonist remaining to be documented pharmacologically.

In this study we utilize for the first time, a mixed MC_3 partial agonist/antagonist (Fig. 1) and MC_4 agonist (JRH322-18) in attempts to further probe the role of the MC_3 in the regulation of food intake in combination with the MC_4 knockout mice that only have intact MC_3s. As anticipated due to its potency and full agonist pharmacology at the MC_4, JRH322-18 resulted in a dose–response decrease in food intake in satiated wild type mice starting at 4 h post icv treatment. In the MC_4KO mice, at 24 h post treatment, a significant ($p<0.05$) decrease in food intake was observed at the 2.0 nmol concentration (Fig. 3). Additionally the control tetrapeptide JRH887-9 (MC_3 and MC_4 full agonist) resulted in decreased food intake in wild type mice, and unexpectedly also in the MC_4KO mice, albeit at 16 h post 2.0 nmol icv treatment. Interestingly, upon treatment in wild type mice of the JRH compounds possessing different *in vitro* MC_3 and MC_3 receptor pharmacological profiles (Table 1), c-Fos-immunoreactivity in the DMH was different (Fig. 4) suggesting that some hypothalamic regions may be stimulated differently, depending upon the ligand pharmacological profile, and thus have variant physiological effects. These data preliminarily support such a hypothesis and further experimental data would need to be generated to either support or disprove this concept.

As the effect of food intake of the JRH compounds in the MC_4 knockout mice was unanticipated based upon the hypothesis that only the MC_4 was involved in the regulation of food intake (Marsh et al., 1999a), we treated the same experimental mice with a gain of function experiment by treatment with the SHU9119 and AGRP ligands demonstrated previously to increase food intake in wild type rodents (Fan et al., 1997; Hagan et al., 2000). While SHU9119 increased food intake in the wild type mice starting at 5 h post treatment that lasted for only a 24 h duration, both the MC_3KO and MC_4KO mice, did not differ in food intake between saline and up to 3.0 nmol SHU9119 concentrations were observed. AGRP however, produced a robust food intake response in the wild type, MC_4KO, and

MC_3KO mice that lasted for several days following a single treatment. These data are consistent with the previous report by Marsh et al. (1999b) showing an increased food intake response in the MC_4KO mice as well as that by Hagen et al. reporting a prolonged duration of action (Hagan et al., 2001) in response to AGRP treatment. However the newly provided data demonstrate that a prolonged response of AGRP in both the MC_3 and MC_4 knockout mice could support the hypothesis that a synergistic as well as independent actions of AGRP on the melanocortin receptors increases food intake. Interestingly, using a rat based activity-based anorexia experimental paradigm, Hillebrand et al. reported that AGRP, but not SHU9119, increased feeding and body temperature (Hillebrand et al., 2006) which also support this hypothesis using a different rodent model and experimental paradigm.

The MC_3 has been postulated to serve as an auto-regulatory factor in a feedback mechanism involving the melanocortin agonists within the arcuate nucleus of the hypothalamus (Cowley et al., 1999). However the role of the MC_3 in other expression sites within the brain and periphery remains to be determined. The use of MC_3 selective ligands and the melanocortin receptor knockout mice are tools that can be used to study the mechanistic and physiological consequences of different receptor expression profiles in distinct places and tissues.

5. Conclusions

There is mounting experimental evidence supporting the hypothesis that the MC_3 is involved in the regulation of food intake, albeit to a lesser extent than the MC_4. However, since the field is lacking specific MC_3 agonists and antagonists, the use of the mixed pharmacology ligands and melanocortin knockout mice has been a key experimental paradigm to probe this hypothesis. To date however, the differences in ligand receptor pharmacology (i.e. MC_3 partial agonist/antagonist) and selectivity profiles have only resulted in confounding results that do not allow for a clear mechanistic model to be put forward. Nonetheless, the data presented herein examine the effect on food intake for the first time on a mixed MC_3 partial agonist/antagonist and MC_4 full agonist in the wild type and MC_4 knockout mice. Further experiments probing the role of the SHU9119 and AGRP antagonists in wild type, MC_3, and MC_4 knockout mice demonstrate physiological differences in food intake responses using ligands with differing MC_3 and MC_3 *in vitro* receptor pharmacological profiles.

Acknowledgments

This work was supported by NIH Grants DK57080 and DK6425 (CHL). Boman Irani is a recipient of an American Heart Association Predoctoral Fellowship. The authors would like to thank Mr. Gabriel Gaidosh for expert guidance and advice on the c-Fos assay and Dr. Glenn Walter for the use of the Leica DC500 imaging system.

References

Atalayer, D., Robertson, K.L., Haskell-Luevano, C., Andreasen, A., Rowland, N.E., 2010. Food demand and meal size in mice with single or combined disruption of melanocortin type 3 and 4 receptors. Am. J. Physiol. Regul. Integr. Comp. Physiol. 298, R1667–R1674.

Benoit, S.C., Sheldon, R.J., Air, E.L., Messerschmidt, P., Wilmer, K.A., Hodge, K.M., Jones, M.B., Eckstein, D.M., McOsker, C.C., Woods, S.C., Seeley, R.J., 2003. Assessment of the aversive consequences of acute and chronic administration of the melanocortin agonist, MTII. Int. J. Obes. Relat. Metab. Disord. 27, 550–556.

Butler, A.A., Kesterson, R.A., Khong, K., Cullen, M.J., Pelleymounter, M.A., Dekoning, J., Baetscher, M., Cone, R.D., 2000. A unique metabolic syndrome causes obesity in the melanocortin-3 receptor-deficient mouse. Endocrinology 141, 3518–3521.

Chen, A.S., Marsh, D.J., Trumbauer, M.E., Frazier, E.G., Guan, X.M., Yu, H., Rosenblum, C.I., Vongs, A., Feng, Y., Cao, L., Metzger, J.M., Strack, A.M., Camacho, R.E., Mellin, T.N., Nunes, C.N., Min, W., Fisher, J., Gopal-Truter, S., MacIntyre, D.E., Chen, H.Y., Van Der Ploeg, L.H., 2000. Inactivation of the mouse melanocortin-3 receptor results in increased fat mass and reduced lean body mass. Nat. Genet. 26, 97–102.

Cowley, M.A., Pronchuk, N., Fan, W., Dinulescu, D.M., Colmers, W.F., Cone, R.D., 1999. Integration of npy, agrp, and melanocortin signals in the hypothalamic paraventricular nucleus: evidence of a cellular basis for the adipostat. Neuron 24, 155–163.

Fan, W., Boston, B.A., Kesterson, R.A., Hruby, V.J., Cone, R.D., 1997. Role of melanocortinergic neurons in feeding and the agouti obesity syndrome. Nature 385, 165–168.

Franklin, K.B.J., Paxinos, G., 1997. The Mouse Brain in Stereotaxic Coordinates. Academic Press, San Diego.

Greenfield, J.R., Miller, J.W., Keogh, J.M., Henning, E., Satterwhite, J.H., Cameron, G.S., Astruc, B., Mayer, J.P., Brage, S., See, T.C., Lomas, D.J., O'Rahilly, S., Farooqi, I.S., 2009. Modulation of blood pressure by central melanocortinergic pathways. N. Engl. J. Med. 360, 44–52.

Grieco, P., Balse, P.M., Weinberg, D., MacNeil, T., Hruby, V.J., 2000. D-amino acid scan of γ-melanocyte-stimulating hormone: importance of trp(8) on human mc3 receptor selectivity. J. Med. Chem. 43, 4998–5002.

Hagan, M.M., Rushing, P.A., Pritchard, L.M., Schwartz, M.W., Strack, A.M., Van Der Ploeg, L.H., Woods, S.C., Seeley, R.J., 2000. Long-term orexigenic effects of agrp-(83–132) involve mechanisms other than melanocortin receptor blockade. Am. J. Physiol. Regul. Integr. Comp. Physiol. 279, R47–R52.

Hagan, M.M., Benoit, S.C., Rushing, P.A., Pritchard, L.M., Woods, S.C., Seeley, R.J., 2001. Immediate and prolonged patterns of agouti-related peptide-(83–132)-induced c-fos activation in hypothalamic and extrahypothalamic sites. Endocrinology 142, 1050–1056.

Haskell-Luevano, C., Monck, E.K., 2001. Agouti-related protein (agrp) functions as an inverse agonist at a constitutively active brain melanocortin-4 receptor. Regul. Pept. 99, 1–7.

Haskell-Luevano, C., Monck, E.K., Wan, Y.P., Schentrup, A.M., 2000. The agouti-related protein decapeptide (yc[crffnafc]y) possesses agonist activity at the murine melanocortin-1 receptor. Peptides 21, 683–689.

Haskell-Luevano, C., Cone, R.D., Monck, E.K., Wan, Y.-P., 2001. Structure activity studies of the melanocortin-4 receptor by *in vitro* mutagenesis: identification of agouti-related protein (agrp), melanocortin agonist and synthetic peptide antagonist interaction determinants. Biochemistry 40, 6164–6179.

Hillebrand, J.J., Kas, M.J., Scheurink, A.J., van Dijk, G., Adan, R.A., 2006. Agrp(83–132) and SHU9119 differently affect activity-based anorexia. Eur. Neuropsychopharmacol. 16, 403–412.

Holder, J.R., Bauzo, R.M., Xiang, Z., Haskell-Luevano, C., 2002. Structure–activity relationships of the melanocortin tetrapeptide Ac-His-DPhe-Arg-Trp-NH_2 at the mouse melanocortin receptors: part 2 modifications at the Phe position. J. Med. Chem. 45, 3073–3081.

Huszar, D., Lynch, C.A., Fairchild-Huntress, V., Dunmore, J.H., Smith, F.J., Kesterson, R.A., Boston, B.A., Fang, Q., Berkemeir, L.R., Gu, W., Cone, R.D., Campfield, L.A., Lee, F., 1997. Targeted disruption of the melanocortin-4 receptor results in obesity in mice. Cell 88, 131–141.

Lee, M., Kim, A., Conwell, I.M., Hruby, V., Mayorov, A., Cai, M., Wardlaw, S.L., 2008. Effects of selective modulation of the central melanocortin-3-receptor on food intake and hypothalamic pomc expression. Peptides 29, 440–447.

Low, M.J., Simerly, R.B., Cone, R.D., 1994. Receptors for the melanocortin peptides in the central nervous system. Curr. Opin. Endocrinol. Diabetes 1, 79–88.

Marks, D.L., Hruby, V., Brookhart, G., Cone, R.D., 2006. The regulation of food intake by selective stimulation of the type 3 melanocortin receptor (MC3R). Peptides 27, 259–264.

Marsh, D.J., Hollopeter, G., Huszar, D., Laufer, R., Yagaloff, K.A., Fisher, S.L., Burn, P., Palmiter, R.D., 1999a. Response of melanocortin-4 receptor-deficient mice to anorectic and orexigenic peptides. Nat. Genet. 21, 119–122.

Marsh, D.J., Miura, G.I., Yagaloff, K.A., Schwartz, M.W., Barsh, G.S., Palmiter, R.D., 1999b. Effects of neuropeptide y deficiency on hypothalamic agouti-related protein expression and responsiveness to melanocortin analogues. Brain Res. 848, 66–77.

McMinn, J.E., Wilkinson, C.W., Havel, P.J., Woods, S.C., Schwartz, M.W., 2000. Effect of intracerebroventricular α-msh on food intake, adiposity, c-fos induction, and neuropeptide expression. Am. J. Physiol. Regul. Integr. Comp. Physiol. 279, R695–R703.

Mountjoy, K.G., Robbins, L.S., Mortrud, M.T., Cone, R.D., 1992. The cloning of a family of genes that encode the melanocortin receptors. Science 257, 1248–1251.

Nijenhuis, W.A., Oosterom, J., Adan, R.A., 2001. AGRP(83–132) acts as an inverse agonist on the human-melanocortin-4 receptor. Mol. Endocrinol. 15, 164–171.

Proneth, B., Pogozheva, I.D., Portillo, F.P., Mosberg, H.I., Haskell-Luevano, C., 2008. Melanocortin tetrapeptide Ac-His-DPhe-Arg-Trp-NH_2 modified at the para position of the benzyl side chain (DPhe): importance for mouse melanocortin-3 receptor agonist versus antagonist activity. J. Med. Chem. 51, 5585–5593.

Thiele, T.E., van Dijk, G., Yagaloff, K.A., Fisher, S.L., Schwartz, M., Burn, P., Seeley, R.J., 1998. Central infusion of melanocortin agonist MTII in rats: assessment of c-fos expression and taste aversion. Am. J. Physiol. 274, R248–R254.

Wessells, H., Fuciarelli, K., Hansen, J., Hadley, M.E., Hruby, V.J., Dorr, R., Levine, N., 1998. Synthetic melanotropic peptide initiates erections in men with psychogenic erectile dysfunction: double-blind, placebo controlled crossover study. J. Urol. 160, 389–393.

Wilczynski, A., Wang, X.S., Joseph, C.G., Xiang, Z., Bauzo, R.M., Scott, J.W., Sorensen, N.B., Shaw, A.M., Millard, W.J., Richards, N.G., Haskell-Luevano, C., 2004a. Identification of putative agouti-related protein(87–132)-melanocortin-4 receptor interactions by homology molecular modeling and validation using chimeric peptide ligands. J. Med. Chem. 47, 2194–2207.

Wilczynski, A.M., Wang, X.S., Bauzo, R.M., Xiang, Z., Shaw, A.M., Millard, W.J., Richards, N.G.J., Edison, A.S., Haskell-Luevano, C., 2004b. Structural characterization of a potent (Cys101–Cys119, Cys110–Cys117) bicyclic agouti-related protein (AGRP) melanocortin receptor antagonist. J. Med. Chem. 47, 5662–5673.