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

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### 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<sub>3</sub> in the regulation of food intake, JRH322-18 a mixed MC<sub>3</sub> partial agonist/antagonist and MC<sub>4</sub> agonist tetrapeptide was examined in wild type (WT) and melanocortin 4 receptor (MC<sub>4</sub>) 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 4hrs post icv treatment into satiated nocturnally feeding wild type mice. The same dose in the MC<sub>4</sub>KO mice significantly reduced cumulative food intake 24h post treatment. Conditioned taste aversion as well as activity studies support 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<sub>3</sub> and MC<sub>4</sub> 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<sub>3</sub> and MC<sub>4</sub> 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<sub>3</sub> and MC<sub>4</sub> knockout mice and it had a prolonged affect for several days. These data support the hypothesis that the MC<sub>3</sub> plays a subtle role in the regulation of food intake, however the mechanism by which this is occurring remains to be determined.

### Keywords

melanotropin; MC3R; MC4R; knockout mice; obesity; food intake; feeding; energy homeostasis; AGRP; SHU9119

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## 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<sub>1</sub>) is clearly linked to the melanocortin agonist effects on pigmentation and animal coat coloration (Mountjoy et al., 1992). The melanocortin 4 receptor (MC<sub>4</sub>) 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<sub>3</sub>) 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<sub>3</sub>/MC<sub>4</sub> knockout mice are extremely obese and hyperphagic (Atalayer et al., 2010) and mounting evidence support the hypothesis regarding a synergistic role between the MC<sub>3</sub> and MC<sub>4</sub> in the regulation of energy homeostasis and perhaps food intake.

The study reported herein was designed to probe the role of the MC<sub>3</sub> in the regulation of food intake and determine if a melanocortin ligand possessing mixed MC<sub>3</sub>/MC<sub>4</sub> agonist/antagonist pharmacology might result in a more desirable anti-obesity therapeutic agent versus a highly selective MC<sub>4</sub> 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  $\alpha$ -MSH and NDP-MSH (Haskell-Luevano et al., 2001; Haskell-Luevano et al., 2000; Holder et al., 2002; Proneth et al., 2008; Wilczynski et al., 2004a; Wilczynski et al., 2004b). Fig. 1 illustrates the functional antagonist pharmacology of agouti-related protein (AGRP), SHU9119, and JRH322-18 at the mouse MC<sub>3</sub>R. The AGRP antagonist does not possess any partial agonist activity at the MC<sub>3</sub>R 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 6h 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<sub>4</sub> knockout mice on a mixed B16/129 background were generously provided by Dr. Dennis Huszar at Millennium Pharmaceuticals (Huszar et al., 1997). The MC<sub>3</sub> knockout mice on a mixed B16/129 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  $\mu$ L in saline) was injected ca 2h 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<sub>4</sub>RKO or MC<sub>3</sub>RKO colonies were observed so the data was combined.

### 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 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  $\mu$ g 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 $\pm$ 0.06, PYY(3-36)=1.35 $\pm$ 0.08,  $p$ <0.001), MC<sub>4</sub>KO (saline=0.46 $\pm$ 0.11, PYY(3-36)=1.41 $\pm$ 0.13,  $p$ <0.001), and MC<sub>3</sub>KO (saline=0.76 $\pm$ 0.09, PYY(3-36)=1.60 $\pm$ 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) was 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 (3X, PBS), incubated for 30 minutes in 0.3% H<sub>2</sub>O<sub>2</sub>, rinsed (3X, PBS), and incubated 1h 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 (3X, PBS). Slices were then transferred to biotinylated goat anti-rabbit antibody for 1 h, rinsed (3X, PBS), and developed with diaminobenzidine substrate (5 minutes). Slices were rinsed (3X, 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 2h 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 0.15M isotonic solution) or drug. On the following day, mice again received 2h 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 seconds. 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 (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  $\pm$  S.E.M.. For statistical analyses, compound concentration over time were 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<sub>4</sub> mice of a mixed MC<sub>3</sub> antagonist and MC<sub>4</sub>R agonist tetrapeptide JRH322-18 and the control MC<sub>3</sub>R and MC<sub>4</sub>R tetrapeptide agonist JRH887-9

It is well established that the MC<sub>4</sub> is directly involved in the regulation of food intake. However, little is understood about the involvement of the MC<sub>3</sub> in food intake or the effects of a mixed MC<sub>3</sub> antagonist and MC<sub>4</sub> agonist in wild type mice. The JRH887-9 control peptide was included as a control peptide due to similar pharmacological effects on food intake due to the structural similarity. The MC<sub>4</sub>KO mice were included since it was previously postulated that the melanocortin based feeding effects were only due to the MC<sub>4</sub> and not the MC<sub>3</sub> (Marsh et al., 1999a), even though the MC<sub>3</sub>KO 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<sub>3</sub>. In the wild type littermate mice, as anticipated based upon the *in vitro* MC<sub>4</sub> agonist pharmacology, JRH887-9 decreased food intake 2h post treatment ( $p < 0.05$ ) at the 2.0 nmol dose (Fig. 3). In the MC<sub>4</sub>KO littermate mice, the 2.0 nmol dose significantly decreased food intake ( $p < 0.001$ ) 16h post treatment. Treatment with the mixed MC<sub>3</sub> antagonist/ MC<sub>4</sub> agonist JRH322-18 resulted in significantly decreased food intake in the wild type mice at 4 h post treatment ( $p < 0.05$ ) and in the MC<sub>4</sub>KO mice, 24h 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<sub>3</sub> and MC<sub>4</sub> receptors (Low et al., 1994). Previous studies using  $\alpha$ -MSH and c-Fos-like immunoreactivity 2h 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 X injection 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<sub>3</sub> 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 hour 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 2h 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 assess visceral illness-like effects and gross behavioral changes mice treated with JRH322-18 and saline were monitored using a locomoter 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<sub>3</sub> and MC<sub>4</sub> antagonists in wild type littermate and the MC<sub>3</sub>KO and MC<sub>4</sub>KO mice

The results of the JRH compounds in the MC<sub>4</sub>KO 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 knock out mice. We hypothesized, based upon previous studies, that in the wild type and MC<sub>3</sub>KO mice that have intact MC<sub>4</sub> receptors would increase their food intake following central AGRP administration. In the MC<sub>4</sub>KO mice, with intact MC<sub>3</sub>s we also anticipated an increased food intake response to AGRP based upon previous results by Marsh et al. (Marsh et al., 1999a; Marsh et al., 1999b). 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, MC<sub>3</sub>KO and MC<sub>4</sub>KO 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 24h post treatment at up to 3.0 nmol concentrations (Fig. 8). Comparison of the average 24h daily intake for mice treated with 2.0 nmol AGRP(87-132) or 3.0 nmol SHU9119 are 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<sub>3</sub>KO mice, which has intact MC<sub>4</sub>s, an increase in food intake was observed for days 1, 2, and 4 post treatment. Interestingly, the MC<sub>4</sub>KO mice, with intact MC<sub>3</sub>s, 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<sub>4</sub>KO, or MC<sub>3</sub>KO mice.

#### 4. Discussion

The MC<sub>4</sub> 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<sub>4</sub>KO 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<sub>4</sub>KO mice (Marsh et al., 1999b). Chen et al. reported that MC<sub>3</sub>KO 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<sup>8</sup>- $\gamma$ <sub>2</sub>-MSH (Grieco et al., 2000), a ca 60-fold mouse MC<sub>3</sub> versus MC<sub>4</sub> selective agonist, stimulates food intake in rats (Lee et al., 2008). Marks et al. (Marks et al., 2006) reported that i.p. administration of D-Trp<sup>8</sup>- $\gamma$ <sub>2</sub>-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 mcg i.p. at 4h post treatment resulted in decreased nocturnal food intake in wild type mice yet the MC<sub>4</sub>KO mice increased food consumption upon treatment. While previous reports in the literature suggest that MC<sub>3</sub> D-Trp<sup>8</sup>- $\gamma$ <sub>2</sub>-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<sub>3</sub> agonists or antagonists (i.e. devoid of any MC<sub>4</sub>R and MC<sub>5</sub>R functional activity) would be a valuable tool to discern the role of the MC<sub>3</sub> in the regulation of food intake, however non exist to date. The  $\gamma$ -MSH related analogues are all full agonists at the MC<sub>3</sub> and MC<sub>4</sub> receptors (albeit with differing degrees of receptor selectivity profiles), the SHU9119 is a MC<sub>3</sub> partial agonist and competitive antagonist (Fig. 1) as well as a potent MC<sub>4</sub> antagonist, and AGRP is a potent MC<sub>3</sub> and MC<sub>4</sub> competitive antagonist as well as an inverse agonist at the MC<sub>4</sub> (Haskell-Luevano and Monck, 2001; Nijenhuis et al., 2001) with its potential as an MC<sub>3</sub> inverse agonist remaining to be documented pharmacologically.

In this study we utilize for the first time, a mixed MC<sub>3</sub> partial agonist/antagonist (Fig. 1) and MC<sub>4</sub> agonist (JRH322-18) in attempts to further probe the role of the MC<sub>3</sub> in the regulation of food intake in combination with the MC<sub>4</sub> knockout mice that only have intact MC<sub>3</sub>s. As anticipated due to its potency and full agonist pharmacology at the MC<sub>4</sub>, 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<sub>4</sub>KO mice, at 24h 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<sub>3</sub> and MC<sub>4</sub> full agonist) resulted in decreased food intake in wild type mice, and unexpectedly also in the MC<sub>4</sub>KO 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<sub>3</sub> and MC<sub>3</sub> 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<sub>4</sub> knockout mice were unanticipated based upon the hypothesis that only the MC<sub>4</sub> 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, in both the MC<sub>3</sub>KO and MC<sub>4</sub>KO 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<sub>4</sub>KO, and MC<sub>3</sub>KO mice that lasted for several days following a single treatment. These data are consistent with the previous report by Marsh et al (Marsh et al., 1999b) showing an increased food intake response in the MC<sub>4</sub>KO 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 a prolonged response of AGRP in both the MC<sub>3</sub> and MC<sub>4</sub> knockout mice could support the hypothesis that a synergistic as well as independent actions of AGRP on the melanocortin receptors increase 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<sub>3</sub> 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<sub>3</sub> in other expression sites within the brain and periphery remain to be determined. The use of MC<sub>3</sub> 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.

## Conclusions

There is mounting experimental evidence supporting the hypothesis that the MC<sub>3</sub> is involved in the regulation of food intake, albeit to a lesser extent than the MC<sub>4</sub>. However, since the field is lacking specific MC<sub>3</sub> 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<sub>3</sub> 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<sub>3</sub> partial agonist/antagonist and MC<sub>4</sub> full agonist in the wild type and MC<sub>4</sub> knockout mice. Further experiments probing the role of the SHU9119 and AGRP antagonists in wild type, MC<sub>3</sub>, and MC<sub>4</sub> knockout mice demonstrate physiological differences in food intake responses using ligands with differing MC<sub>3</sub> and MC<sub>3</sub> *in vitro* receptor pharmacological profiles.

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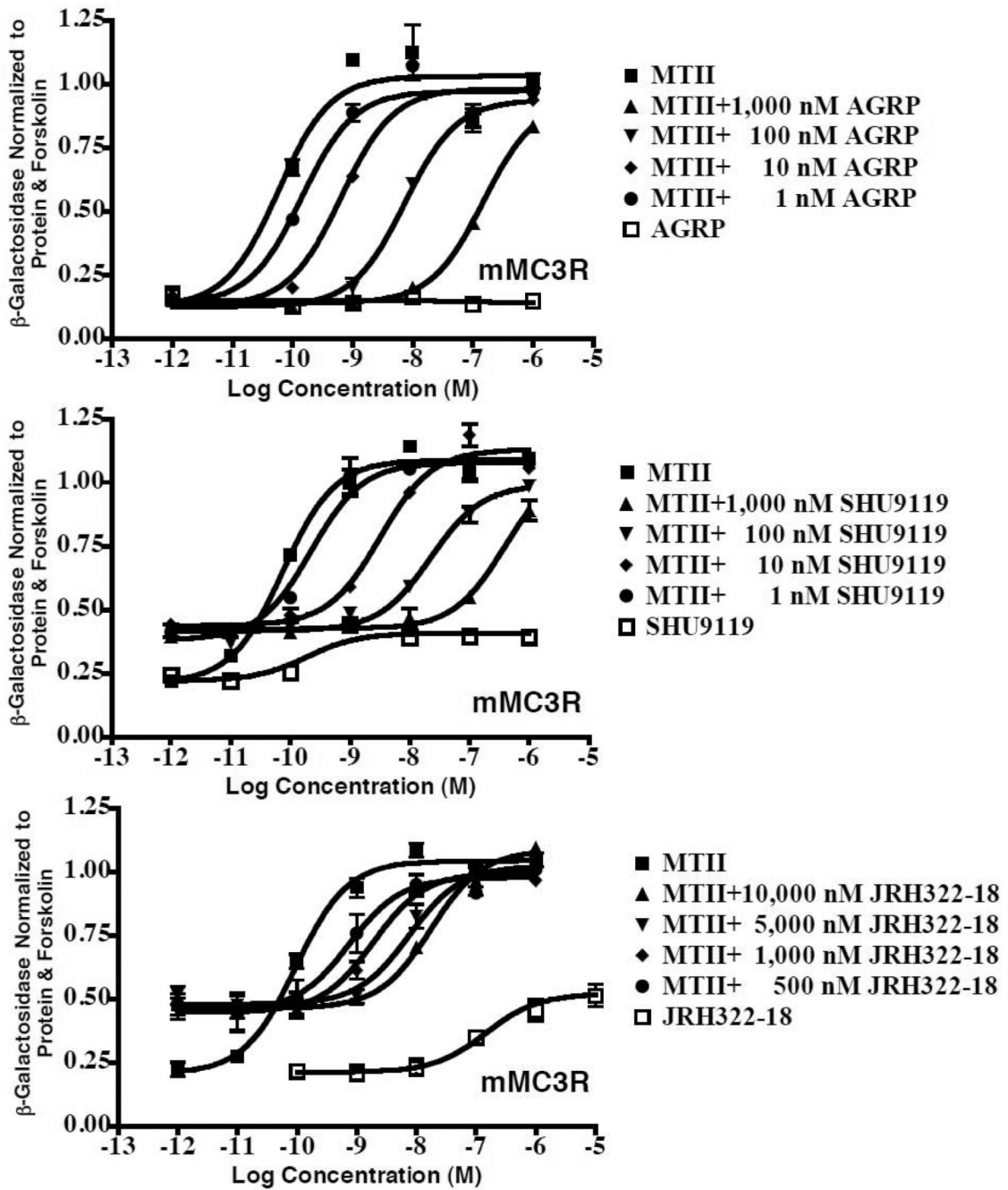
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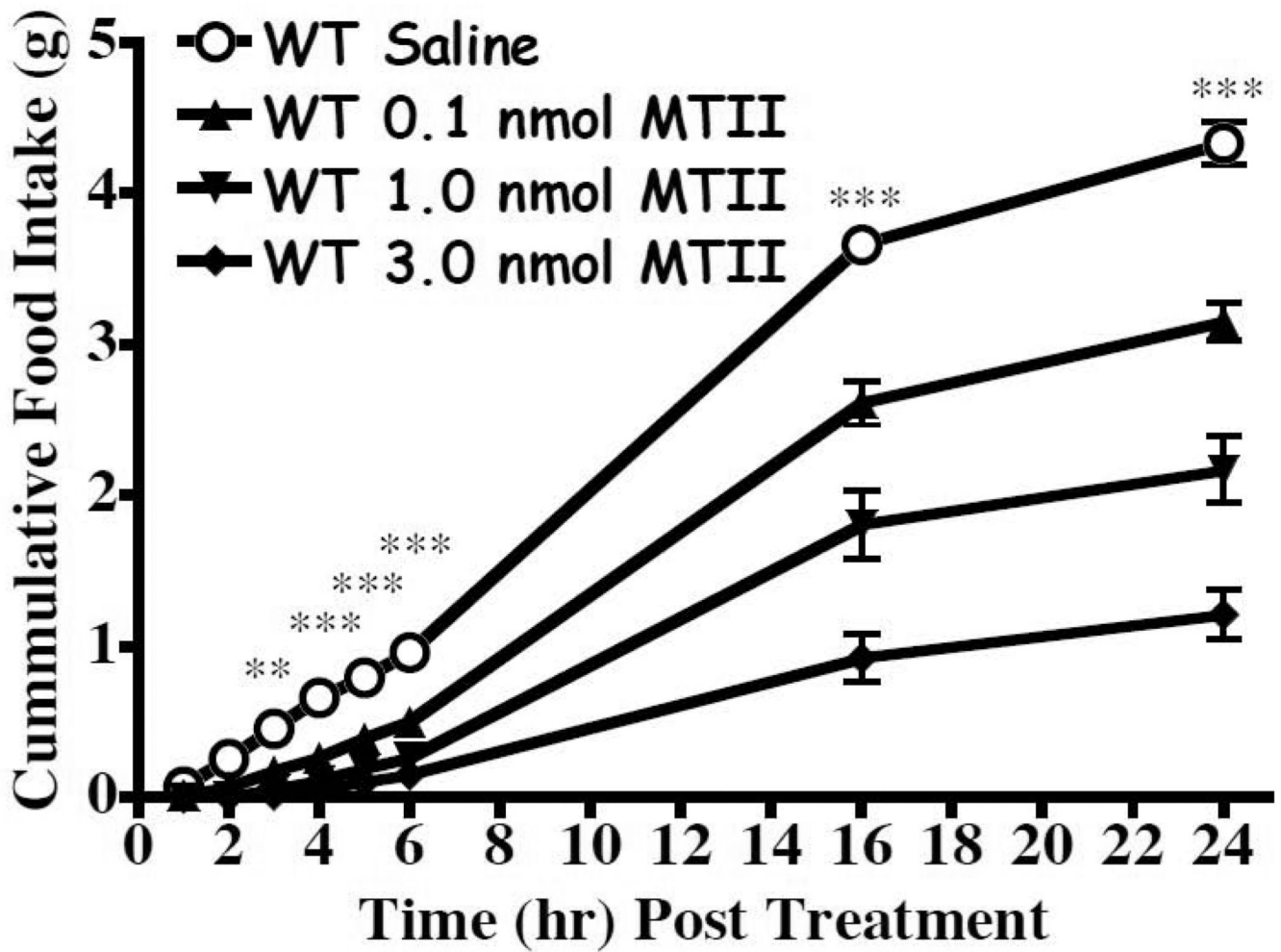
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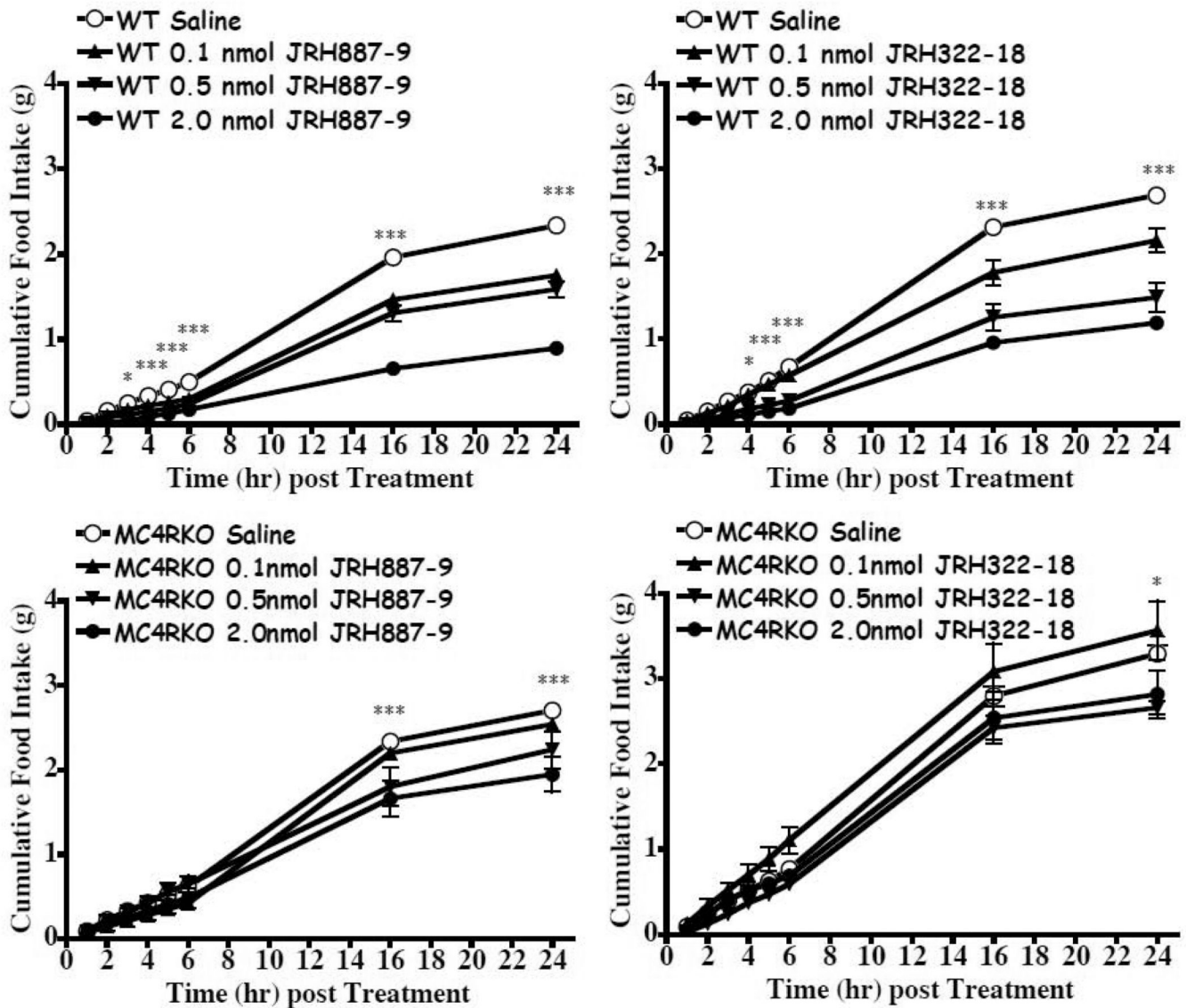
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**Figure 1.**  
*In vitro* mouse MC3 receptor pharmacology of the AGRP, SHU9119, and JRH322-18 peptides examined in this study.

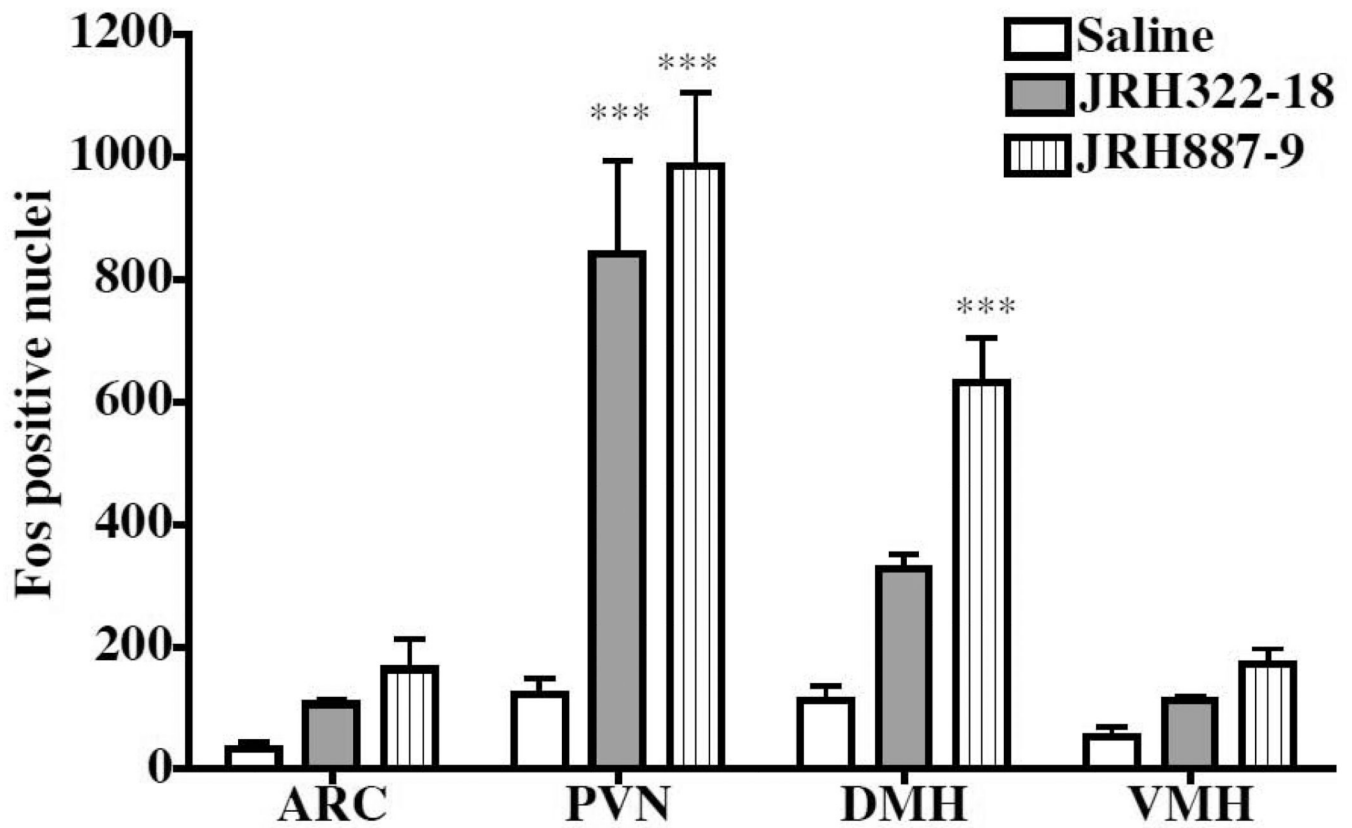


**Figure 2.** Effect of MTII on food intake (mean  $\pm$  S.E.M.) in wild type mice after lateral ventricular administration using a non-fasted paradigm ( $p < 0.0001$   $n = 17/\text{group}$ ). The 0.1, 1.0, and 3.0 nmol doses statistically decreased food intake relative to saline starting at  $t =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

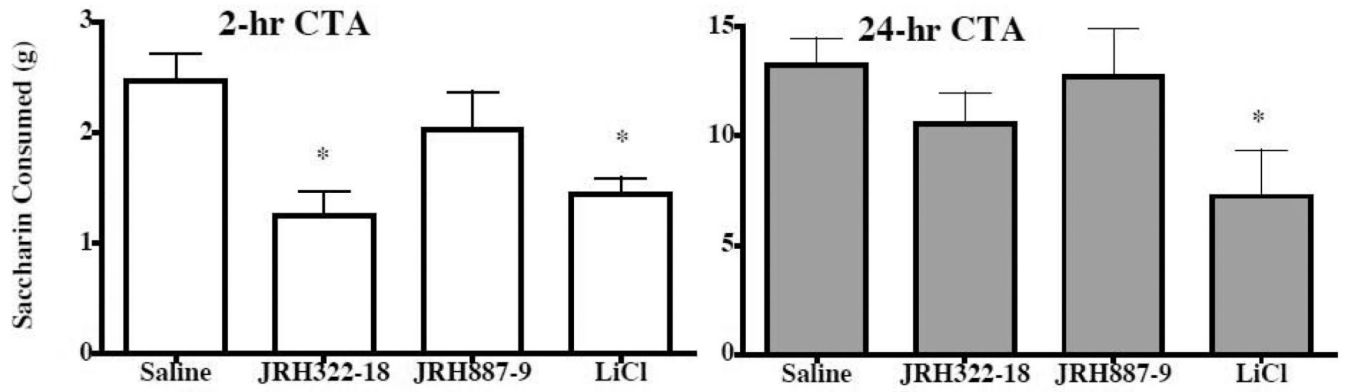


**Figure 3.**

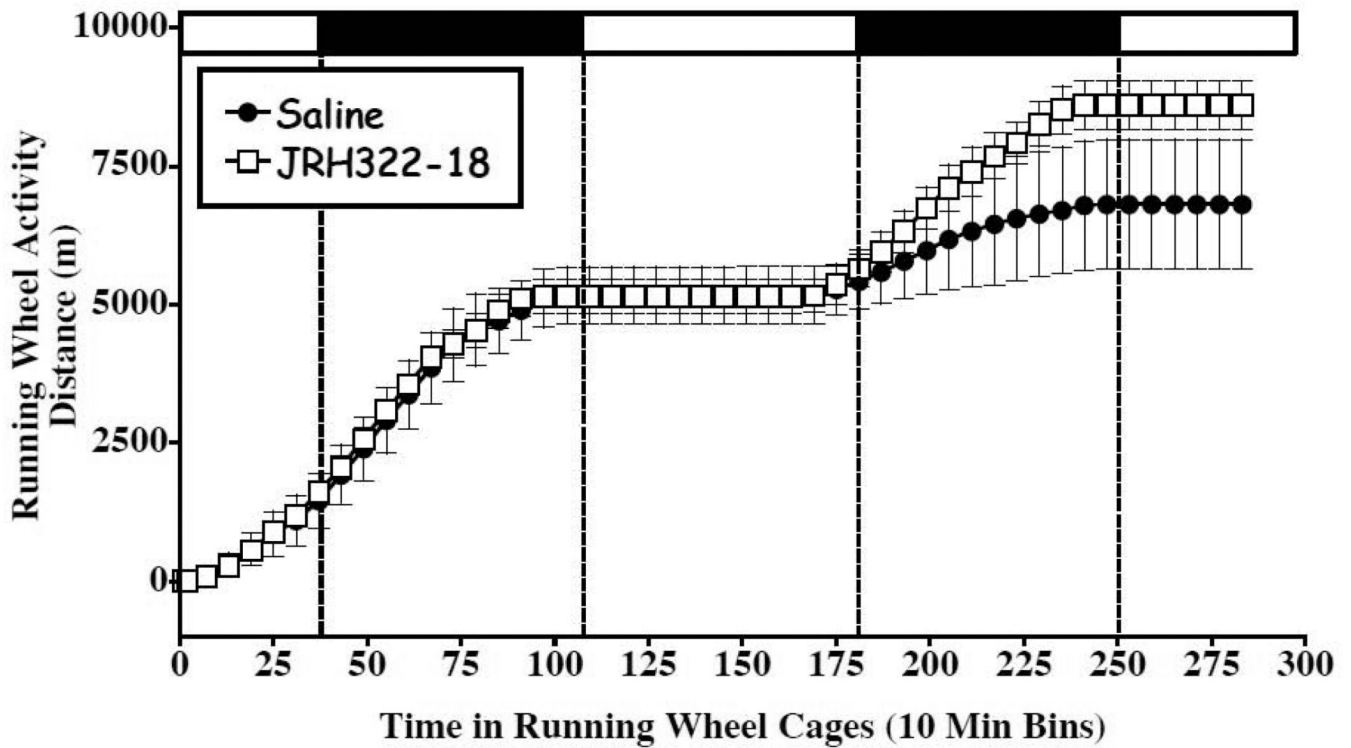
Effect of the JRH tetrapeptides on food intake (mean  $\pm$  S.E.M.) in the wild type and MC4KO littermate mice (n=10–12 per group wild type and n=6 per group for the MC4KO mice). These compounds significantly decreased food intake at the wild type and MC4KO mice ( $p < 0.0001$ ). The tetrapeptide JRH887-9 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=5h, t=4h, and t=3h, respectively ( $*p < 0.05$ ). In the MC4KO mice, the 0.5, and 2.0 nmol doses statistically decreased food intake relative to saline starting at t=16h ( $***p < 0.001$ ). 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=16h, t=5h, and t=4h, respectively ( $p < 0.05$ ). In the MC4KO mice, the 0.5 and 2.0 nmol doses statistically decreased food intake relative to saline starting at t=24h ( $p < 0.05$ ).



**Figure 4.** Effect of JRH treatment on hypothalamic c-Fos-like immunoreactivity in wild type mice 2h 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 MC3 antagonist as well as a MC4 agonist.

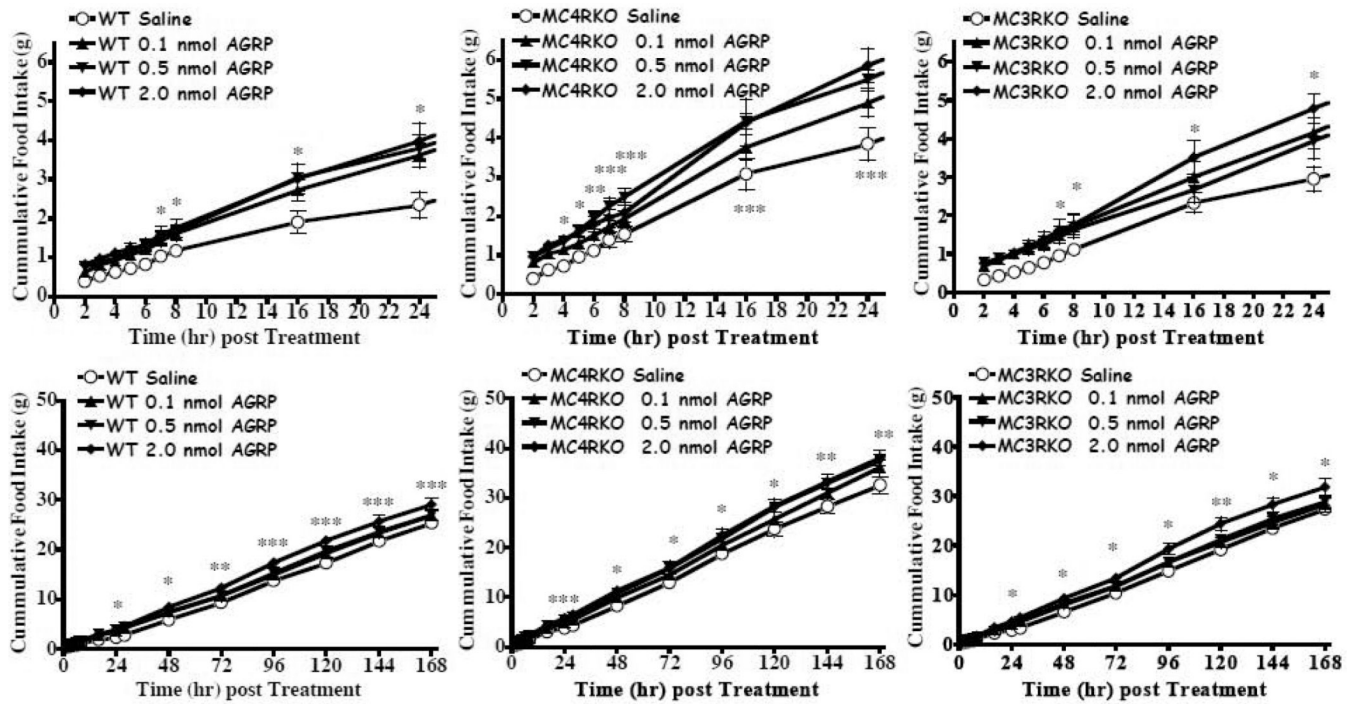


**Figure 5.** Effect of JRH treatment (1.0 nmol) in wild type mice in conditioned taste aversion studies (n=4–5 mice per group). At the 2h time point, a difference between saline and JRH322-18 (\*p<0.05) treatment in the amount of saccharin consumed is observed. However, it is not observed at the 24hr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



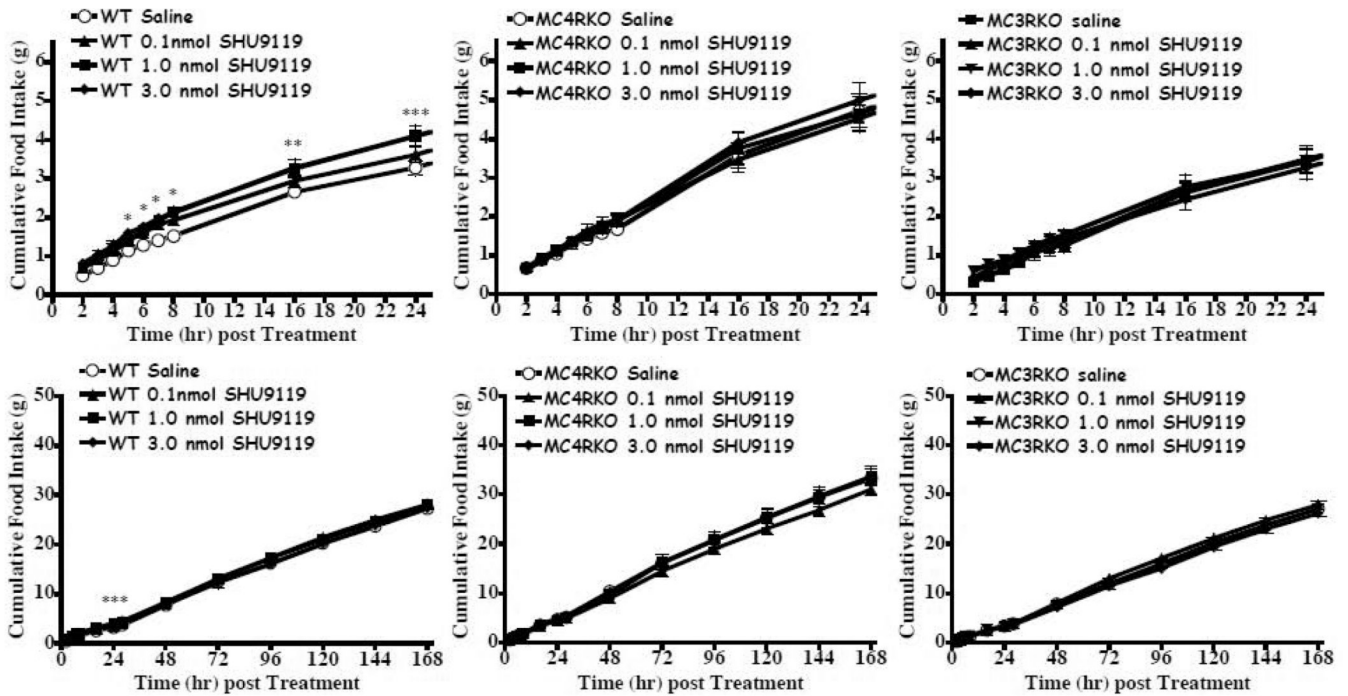
**Figure 6.**

Locomotor assay to assess visceral illness-like effects and gross behavioral changes of JRH322-18 relative to saline treatment in wild type mice. Mean  $\pm$  S.E.M. distance traveled (in meters) during 48h in running wheel cages for mice injected into the 3<sup>rd</sup> ventricle with 1 $\mu$ l 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, Y-axis is distance traveled in meters. Dashed vertical lines show transition points during light-dark cycle, 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.

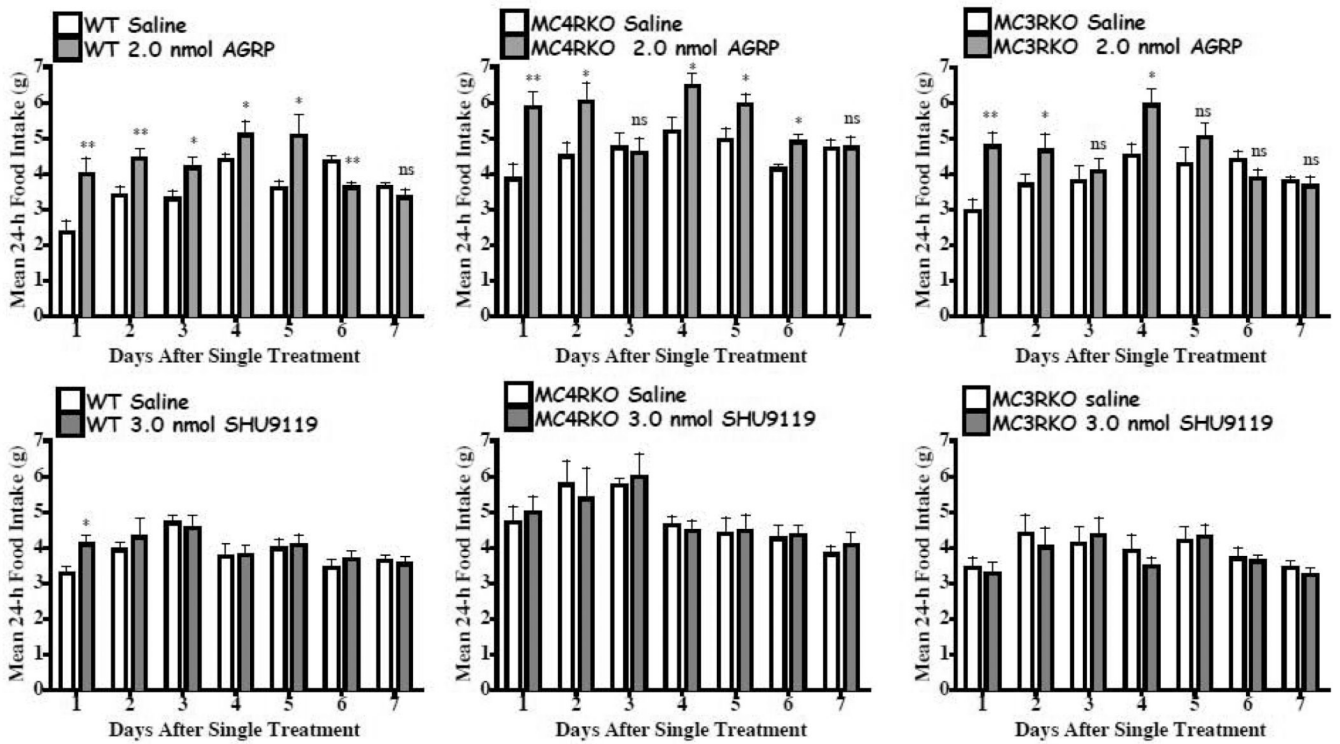


**Figure 7.** Effect of AGRP(86-132) on food intake (mean  $\pm$  S.E.M.) in satiated wild type, MC4KO, and MC3KO littermate mice. For all the mice, statistically increased food intake was observed relative to the saline control ( $p < 0.0001$ ,  $n = 8-9$  per group). A) In the wild type mice the 2.0 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 MC4KO 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 MC3KO 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 ( $*p < 0.05$ ). \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$





**Figure 8.** Effect of SHU9119 on food intake (mean  $\pm$  S.E.M.) in satiated wild type, MC4KO, and MC3KO littermate mice (n=10–12 per group). A) In the wild type mice the 1.0 and 3.0 nmol dose statistically increased food intake relative to saline starting at t=7h and t= 5h, respectively, and continued to remain statistically significant up to the 24h post injection time point (\*p<0.05). B) In the MC4KO and MC3KO mice, no statistically significant changes in food intake relative to saline was observed.



**Figure 9.** Shows the average daily food intake (mean ± S.E.M.) per 24h after a single injection of either AGRP(86-132) or SHU9119 (same groups as in Figures 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, ns=not statistically different.

Table 1

Summary of *in vitro* mouse melanocortin receptor pharmacology.

Name	Structure	Functional Activity (nM) mM3R	Binding Affinity (nM) mM3R	Functional Activity (nM) mM4R	Binding Affinity (nM) mM4R
$\alpha$ -MSH	Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH <sub>2</sub>	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 <sub>2</sub>	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 <sub>2</sub>	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 <sub>2</sub>	Antagonist* 0.32	3.2±1.1	Antagonist 0.040	0.38±0.10
JRH887-9	Ac-His-DPhe-Arg-Trp-NH <sub>2</sub>	Agonist 160±9.2	ND	Agonist 17±2.8	ND
JRH322-18	Ac-His-(p)DPhe-Arg-Trp-NH <sub>2</sub>	Antagonist* 50±1.1	270±146	Agonist 25±9.8	4.81±4.5
AGRP(87-132)		Antagonist 1.4	2.8±0.4	Antagonist 0.41	3.0±0.3

ND indicates values not reported.

\* Indicates that the antagonists also possess partial agonist activity at the mM3R. The indicated error represents the standard error of the mean.