In vivo evidence for inverse agonism of agouti related peptide in the central nervous system of proopiomelanocortin deficient mice

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Running Title: AgRP actions in POMC-deficient mice

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ABSTRACT

OBJECTIVE: Melanocyte stimulating hormone (MSH) peptides processed from proopiomelanocortin (POMC) regulate energy homeostasis by activating neuronal melanocortin receptor (MC-R) signaling. Agouti related peptide (AgRP) is a naturally occurring MC-R antagonist, but also displays inverse agonism at constitutively active MC4-R expressed on transfected cells. We investigated whether AgRP functions similarly in vivo using mouse models that lack all neuronal MSH, thereby precluding competitive antagonism of MC-R by AgRP.

RESEARCH DESIGN AND METHODS: Feeding and metabolic effects of the MC-R agonist MTII, AgRP, and ghrelin were investigated after icv injection in neural-specific POMC-deficient (Pomc-/-Tg/+) and global POMC-deficient (Pomc-/-) mice. Gene expression was quantified by RT-PCR.

RESULTS: Hyperphagic POMC-deficient mice were more sensitive than wildtype mice to the anorectic effects of MTII. Hypothalamic MC3/4-R mRNAs in POMC-deficient mice were unchanged, suggesting increased receptor sensitivity as a possible mechanism for the heightened anorexia. AgRP reversed MTII-induced anorexia in both mutant strains, demonstrating its ability to antagonize MSH agonists at central MC3/4-R, but did not produce an acute orexigenic response by itself. Ghrelin’s action was attenuated in Pomc-/-Tg/+ mice, suggesting decreased sensitivity to additional orexigenic signals. However, AgRP induced delayed and long-lasting modifications of energy balance in Pomc-/-Tg/+, but not glucocorticoid-deficient Pomc-/- mice, by decreasing oxygen consumption, increasing the respiratory exchange ratio, and increasing food intake.

CONCLUSIONS: These data demonstrate that AgRP can modulate energy balance via a mechanism independent of MSH and MC3/4-R competitive antagonism, consistent with either inverse agonist activity at MC-R or interaction with a distinct receptor.

Key words: Agouti Related Peptide (AgRP), Proopiomelanocortin (POMC), melanocortin receptors (MC-R), melanotan II (MTII), inverse agonism, food intake, indirect calorimetry

Nonstandard abbreviations used: Agouti Related Peptide (AgRP); arcuate nucleus (Arc); Cocaine and Amphetamine Related Transcript (CART); diet-induced obesity (DIO); melanocortin receptor (MC-R); melanocortin-4 receptor (MC4-R); melanocortin-3 receptor (MC3-R); melanocyte stimulating hormone (MSH); proopiomelanocortin (POMC); respiratory exchange ratio (RER)
Genetic disruption of either mouse or human proopiomelanocortin (POMC) causes early-onset obesity (1-3), highlighting a major role of POMC in the regulation of energy homeostasis. POMC is processed post-translationally into multiple peptides, including the opioid β-endorphin and the melanocortins ACTH, α-melanocyte stimulating hormone (αMSH), βMSH, and γMSH. POMC peptides in the CNS are essential in the regulation of energy intake and expenditure as demonstrated in studies using compound mutant mice (Pomc<sup>-/-</sup>Tg/+<sup>+</sup>) expressing a Pomc transgene that selectively restored pituitary POMC in Pomc<sup>-/-</sup> mice to produce a neural-selective POMC-deficiency (4). Lack of α-MSH is likely the principal cause of obesity (3, 5) due to the loss of agonist signaling at central melanocortin receptors (MC-R), MC3-R and MC4-R, each of which plays a distinct role in the regulation of energy homeostasis (6-8).

The anorectic actions of centrally administered αMSH or the synthetic MC3/4-R agonist MTII (9-11) are blocked by Agouti Related Peptide (AgRP), an endogenous MC3/4-R antagonist (12, 13), released from terminals of neuropeptide Y/AgRP arcuate neurons. In addition to their localization to the same brain regions as POMC fibers (14), AgRP nerve terminals send projections to neurons that possess MC4-R (15) but are not innervated by αMSH terminals (14, 16). These neuroanatomic findings indicate that AgRP may modulate MC4-R activity in the absence of endogenous αMSH.

*In vitro* data strongly support the ability of AgRP to modulate MC4-R by an inverse agonist mode of action (17-20), however the physiological significance is unresolved. Modulation of MC4-R constitutive activity may be important to maintain long-term energy homeostasis in humans (21). In rodents, the concept of inverse agonism has been buttressed by demonstrations that a single injection of AgRP induces hyperphagia over several days (22-24) whereas this long-lasting effect cannot be reproduced by synthetic MC4-R antagonists like HS014 or JKC-363 (23, 25).

In the present study, we analyzed the feeding and metabolic responses to intracerebroventricular (icv) injections of MTII and AgRP in mice deficient in all central MSH peptides. Because responses to melanocortin antagonists apparently require the presence of circulating glucocorticoids (26), we compared Pomc<sup>-/-</sup> mice with a global deficiency of POMC and adrenal insufficiency to Pomc<sup>-/-</sup>Tg/+<sup>+</sup> mice with a neural-specific deficiency of POMC but restored glucocorticoids (4). Feeding effects of the orexigenic gut peptide ghrelin (27) were also tested.

**RESEARCH DESIGN AND METHODS**

**Animals.** A colony of Pomc mutant mice on a hybrid B6;D2;129X1;129S6 genetic background with independently segregating Pomc<sup>-/-</sup> and pHalEx2* Tg alleles was established as described previously (4). Mice were maintained under controlled temperature and photoperiod (12 h light, 12 h dark; lights on at 07:00) with free access to water and chow (4.5% fat, 20% protein, 6% fiber, 3.4 kcal/g; PicoLab Rodent Diet 20, PMI Nutrition International, St. Louis, MO). Experimental procedures were approved by the Institutional Animal Care and Use Committee and followed Public Health Service guidelines.

**Peptides.** MTII, hAgRP (83-132), mAgRP (82-131), and rGhrelin were purchased from Phoenix Pharmaceuticals (Mountain View, CA) and dissolved in physiological saline.
ICV cannulation. Mice were anesthetized with 2% Avertin. 26-gauge stainless steel guide cannulae cut 2.5 mm below the pedestal (Plastics one, Roanoke, VA) were implanted stereotaxically into the right lateral ventricle (posterior -0.4 mm, lateral -1.0 mm, relative to bregma), secured to the skull using cap screws (Small Parts Inc.) and dental cement, and occluded with stainless steel dummy obturators. Mice were then housed individually for 7-10 days of recovery without specific treatment, except the Pomc-/- mice that required injection with dexamethasone (0.15 µg ip in 1 ml saline) for 3 days.

Feeding and basal metabolic rate. Peptides were injected icv in a volume of 1 µl over 1 min using a 33-gauge stainless steel injection cannula extending 0.5 mm below the guide cannula and connected to a 1 µl Hamilton syringe with polyethylene tubing. Mice were returned to their home cage and the remaining food in containers on the cage floor was weighed at different time intervals. Basal metabolic rate was determined by indirect open-circuit calorimetry (Oxymax, Columbus Instruments) as previously described (4). After three days of chamber habituation, six measurements were recorded daily from each mouse at 60 min intervals. Individual basal oxygen consumption (VO₂) levels were established by averaging the two lowest VO₂ measurements. Respiratory exchange ratios (RER) were recorded as the molar ratio of VCO₂ to VO₂ and a daily average value was calculated.

Experimental Design. Male and female mice were used in all experiments. Peptides or saline were administered between 11:00 am and 12:00 pm for day-time experiments and between 6:00 and 7:00 pm (lights-off at 7:00 pm) for night-time experiments. Mice were randomized into different groups based on their 24 h food intake before experiments and received each dose of peptide and saline in a counterbalanced order. Detailed designs for each experiment and the number of animals per group are in the Online Appendix (available at http://diabetes.diabetesjournals.org).

Real-time RT-PCR. Mice were decapitated between 9:00-11:00 am and hypothalami dissected on ice, harvested in Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA) and extracted according to the manufacturer’s directions. PCR reactions were performed on an ABI Prism 7300 Sequence Detection System instrument (Perkin-Elmer Applied Biosystem, Foster City, CA) using TaqMan® Gene Expression Assays containing a set of sequence-specific primers and a 6-FAM dye-labeled TaqMan® MGB probe for MC4-R, MC3-R, AgRP, NPY or Cocaine and Amphetamine Related Transcript (CART) and the TaqMan® endogenous control 18S. cDNA samples obtained from reverse transcription of 1 µg RNA were run in duplicate in total reaction volumes of 20 µl containing 5 µl cDNA, 1X TaqMan® Gene Expression Assay and 1X TaqMan® Universal Master Mix. Thermal cycling conditions included an initial denaturation step at 95°C for 10 min followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Real-time PCR data were analyzed with the 2^{-ΔΔCT} method as previously described (28).

Statistical analyses. All data presented are the mean ± SEM. Data were analyzed by repeated measured ANOVAs or multifactor ANOVAs appropriate for the design of each experiment with genotype and/or drug as independent variables using Stat View Power PC for Macintosh version 5.0.1 (SAS Institute Inc.). One factor ANOVAs were used to follow up significant main effects, post hoc pair wise comparisons between groups were performed by Fisher’s protected least squares difference (PLSD) or paired two-tail T-Tests. P values < 0.05 were considered significant.
RESULTS

Feeding effects of MTII injected at the onset of the dark cycle. A single icv injection of 0.5 nmol MTII at the onset of the dark cycle decreased food intake in control Pomc+/+Tg/+ and mutant Pomc++/Tg/+ and Pomc-/- mice compared to vehicle treated animals 2h after the injection (Figure 1A). In Pomc+/+Tg/+ mice, food intake was inhibited 67% and the effect was completely reversed by 24h (Figure 1A and B). In both Pomc-/-Tg/+ and Pomc-/ mice, MTII acutely decreased food intake by 95% and in contrast to control mice, this effect was sustained with 60% and 54% cumulative reductions at 24h (P<0.01 vs saline). Long-term anorexia induced by 0.5 nmol MTII was also associated with significant weight loss in Pomc+/+Tg/+ and Pomc-/- mice (P<0.01 and P<0.05 vs saline, respectively) (Figure 1C).

Feeding effects of co-administered MTII and AgRP. To test the ability of AgRP to antagonize MSH agonists at MC3/4-R, 0.5 nmol AgRP was injected alone or in combination with 0.1 nmol MTII at the onset of the dark cycle. Although this lower dose of MTII was slightly less potent than 0.5 nmol to reduce feeding 2h after injection, we chose it in combination with AgRP because its anorectic effects had dissipated in all genotypes at 24h. There was a significant main effect of the peptide treatments on 2h food intake but no significant interaction of treatment x genotype (Figure 2A). Post-hoc analyses collapsed across genotype confirmed that 0.1 nmol MTII alone significantly decreased acute food intake (P<0.0001 vs saline). In contrast, there was no significant main effect of treatment or treatment x genotype interaction on 24h food intake (Figure 2B). After night-time injection, when maximum feeding activity is already observed, there was no additive, short-term orexigenic effect of 0.5 nmol AgRP alone on any genotype. However, AgRP significantly blocked the anorectic effect of 0.1 nmol MTII in all genotypes 2h after co-injection of both peptides (P<0.0001, AgRP+MTII vs MTII alone, Fisher PLSD) (Figure 2A), consistent with a competitive antagonist action at the MC3/4-R.

Short-term feeding effects of AgRP and ghrelin injected during the light cycle. Administration of 0.5 nmol or 2 nmol AgRP during the day-time increased 2h food intake by 160% and 250%, respectively, in Pomc+/+Tg/+ mice (P<0.05 vs saline). In Pomc-/-Tg/+ mice, neither dose of AgRP had short-term orexigenic effects (Figure 3A, data with 2 nmol not shown). To test the ability of Pomc-/- and Pomc-/-Tg/+ mice to respond to other orexigenic signals, 1 nmol ghrelin was injected during the day-time (Figure 3B). Ghrelin stimulated 2h food intake in Pomc+/+Tg/+ and, to a much lesser extent, in Pomc-/-Tg/+ mice (P<0.0001 and P<0.05 vs saline, respectively), but had no effect in Pomc-/- mice.

Short and long-term effects of AgRP injected at the onset of the dark cycle on food intake and body weight. Night-time injection of 0.5 nmol AgRP, when maximum feeding activity occurs naturally, did not further increase 2h food intake in any genotype (Figure 4A, D, G and J). However, hyperphagia was observed 24h after the injection of AgRP, was sustained up to 72h and accompanied by body weight gain in Pomc+/+Tg/+ mice (Figure 4B and C). Pomc-/- mice were more sensitive to the long-term effects of AgRP with sustained hyperphagia up to 96h post-injection and more pronounced body weight gain than Pomc+/+Tg/+ mice (Figure 4K and L). Neither Pomc-/-Tg/+ nor Pomc-/- mice increased their food consumption after night-time injection of 0.5 nmol AgRP (Figure 4E and H). Furthermore, injection of 0.5 nmol or
2 nmol AgRP during the light cycle also did not produce any long-lasting orexigenic effect in the mutant Pomp^{c/-}Tg/+ mice while it was orexigenic in Pomp^{+/+}Tg/+ control mice (data not shown). Similar to Pomp^{+/+}Tg/+ controls, the rate of body weight gain was greater in Pomp^{c/-}Tg/+ mice injected with 0.5 nmol AgRP than with saline and this difference was significant at 72h (P<0.001) (Figure 4F).

Although a short-lived reduction in food intake and body weight was sometimes observed following icv injections of saline in Pomp^{+/+}Tg/+ controls, the rate of body weight gain was greater in Pomp^{c/-}Tg/+ mice injected with 0.5 nmol AgRP than with saline and this difference was significant at 72h (P<0.001) (Figure 4F).

Effect of AgRP injected at the onset of the dark cycle on VO₂ and RER in mice with restricted food access. To test the hypothesis that AgRP may be a long-term modulator of energy expenditure in Pomp^{c/-}Tg/+ mice, we measured VO₂ and RER during 7 consecutive days following the injection of saline or AgRP at the onset of the dark cycle (Figure 5), conditions that induced weight gain in both Pomp^{+/+}Tg/+ and Pomp^{c/-}Tg/+ mice.

In most Pomp^{+/+}Tg/+ mice, as depicted in two representative individuals (Figure 5A), the effects of AgRP were observed up to 72h following the injection. In contrast, the onset of AgRP effects were delayed by 24h, but subsequently lasted longer in Pomp^{c/-}Tg/+ mice (Figure 5B). Consequently, changes were subtler but more prolonged in Pomp^{c/-}Tg/+ compared to Pomp^{+/+}Tg/+ mice.

Analyses performed for the relevant, genotype-specific time-frames showed an increased body weight of 12% over 3d in Pomp^{+/+}Tg/+ (P<0.01) and 6% over 6d in Pomp^{c/-}Tg/+ (P<0.01) mice after AgRP treatment, compared to their initial body weights. In Pomp^{+/+}Tg/+ mice, increased daily food consumption, increased average daily RER and decreased VO₂ were observed within 24h following the injection (0-24h) and were sustained up to 72h after the injection (24-72h) (Figure 5C, E and G). In contrast, none of the parameters were modified within 24h following AgRP injection (0-24h) in Pomp^{c/-}Tg/+ mice, but the delayed effects on food intake, VO₂ and RER were significant between 24-72h and persisted up to 168h (Figure 5D, F and H).

MC3/4-R, AgRP, NPY, and CART gene expression in the hypothalamus. Expression levels of MC4-R (Figure 6A) and MC3-R (Figure 6B) from the whole hypothalamus were unchanged in Pomp^{c/-}Tg/+ and Pomp^{+/-} mice compared to control Pomp^{+/+}Tg/+ mice.

Unlike MC3/4-R, levels of AgRP (Figure 6C), NPY (Figure 6D) and CART (Figure 6E) mRNA all differed by genotype. In Pomp^{c/-} mice, AgRP expression was significantly decreased by 64%, CART expression was increased by 47%, and NPY expression was unchanged compared to Pomp^{+/+}Tg/+ mice. In Pomp^{c/-}Tg/+ mice, CART expression was increased by 67% but AgRP and NPY were unchanged compared to Pomp^{+/+}Tg/+.

Notably however, in Pomp^{c/-}Tg/+ mice both AgRP and NPY expression were significantly increased compared to the glucocorticoid-deficient Pomp^{c/-} mice.

DISCUSSION

Catabolic effects of MTII were accentuated in obese POMC-deficient mice. Both strains of POMC-deficient mice were more sensitive to the short-term anorectic action of MT-II than their control siblings. The mechanism of increased sensitivity to MTII in POMC-deficient mice or other animal models of obesity, like Zucker rats (fa/fa) (29), diet-induced obesity (DIO) rats (30), and DIO and ob/ob mice (31) with decreased POMC
AgRP actions in POMC-deficient mice

expression or decreased melanocortin tone (32-34), may involve increased expression, density or functional coupling of MC-R in response to the chronic absence or decrease of endogenous melanocortin ligands. There were no significant differences in the expression of MC4-R or MC3-R in Pomc<sup>−/−</sup>Tg/+ and Pomc<sup>−/−</sup> mice compared to Pomc<sup>+/+</sup>Tg/+ mice, suggesting that either increased MC-R density or signaling are responsible for the heightened anorectic effect of MTII. However, our quantification did not take into account differential regional expression of the MC4-R in various hypothalamic nuclei, or extrahypothalamic expression of MC4-R (15) that are important in the regulation of energy homeostasis and have been shown to mediate the effects of melanocortin agonists and antagonists and long-term orexigenic actions of AgRP (35).

Increased sensitivity to MTII could also be due to secondary alterations in the expression of other hypothalamic orexigenic/anorectic signals. AgRP mRNA was reduced in Pomc<sup>−/−</sup> mice, consistent with data reported by Coll and collaborators (36) and supporting the hypothesis that reduced levels of AgRP contributed to the increased response to MTII. However, this cannot be the only explanation as AgRP expression was almost normalized in glucocorticoid-replete Pomc<sup>−/−</sup>Tg/+ mice, which still displayed an exaggerated anorectic response to MTII.

**Differential short- and long-term effects of AgRP in neural selective POMC-deficient mice.** AgRP did not alter short-term food intake but was able to antagonize the anorectic effect of MTII in Pomc<sup>−/−</sup>Tg/+ mice, indicating that the short-term orexigenic effect of AgRP requires the presence of αMSH and therefore is due to a competitive antagonist action at MC3/4-R. Despite re-expression of POMC in the pituitary gland, Pomc<sup>−/−</sup>Tg/+ mice had undetectable levels of αMSH in the hypothalamus (4), excluding the possibility that αMSH from a peripheral source leaked into the CNS. Interestingly, Pomc<sup>+/+</sup> mice had stronger feeding responses to both AgRP and MTII than control Pomc<sup>+/−</sup>Tg/+ mice, probably reflecting a gene-dosage effect in the response to melanocortin agonists and antagonists. We previously demonstrated that the hypothalamic content of αMSH in Pomc<sup>+/−</sup> mice is half that of Pomc<sup>+/+</sup>Tg/+ mice (4), indicating that there is less endogenous αMSH to antagonize in those animals.

In vitro studies have clearly demonstrated that AgRP acts as both a competitive antagonist and an inverse agonist at MC4R and MC3-R to modulate cAMP levels (17-20, 37). Furthermore, in a recent study, AgRP was shown to exhibit agonistic properties on both MC3-R and MC4-R expressed in HEK293 cells by inducing arrestin-mediated endocytosis (38), supporting the inverse agonist hypothesis. However, these alternative signaling properties of AgRP have never been directly demonstrated in vivo. Here we show that in addition to its role as a MC3/4-R competitive antagonist, AgRP is able to modulate energy homeostasis independently of the presence of the endogenous agonist αMSH.

The mechanism of AgRP-induced weight gain in Pomc<sup>+/−</sup>Tg/+ mice involves a long-lasting increase in food consumption and RER and a decrease in energy expenditure. In Pomc<sup>−/−</sup>Tg/+ mice with ad libitum access to food over the 24h period, we were not able to measure any difference in food consumption after AgRP treatment. The observation that exogenous AgRP can induce weight gain without affecting food consumption in certain physiological conditions (when animals were fed ad libitum in our study) is surprising considering the powerful orexigenic properties of the peptide. Nevertheless, one
should consider that feeding responses clearly depend on the endogenous neuropeptide tone involved in the regulation of energy balance. Because Pomc<sup>−/−</sup>Tg/+ mice are constitutively hyperphagic and have increased mRNA levels for the orexigenic peptides AgRP and NPY compared to Pomc<sup>−/−</sup> mice, increased daily food consumption over the heightened baseline may be difficult to detect or to induce. Although Pomc<sup>−/−</sup>Tg/+ mice were still able to respond to the short-term orexigenic effects of ghrelin, which are partly mediated via increased NPY and AgRP tone (27), the percentage of increase was much lower than in Pomc<sup>+/+Tg/+</sup> mice, suggesting that they are less sensitive to stimulation by orexigenic signals. Either pharmacological or physiological manipulations to reduce the basal hyperphagia might be useful to reveal a stronger orexigenic response to exogenous AgRP in the mutant mice.

The reduced food consumption of Pomc<sup>−/−Tg/+</sup> and particularly Pomc<sup>−/−</sup> mice following saline injections indicates a contribution of stress-related feeding responses after icv injections. Our previous data demonstrated a central dysregulation of the HPA axis in Pomc<sup>−/−Tg/+</sup> mice, characterized by inappropriately high hypothalamic CRH levels (39). Here, we show that CART, another factor involved in the control of the HPA axis and possessing anorectic effects (40), is also up-regulated in POMC-deficient mice. Therefore, elevated CRH and CART tone may counterbalance the feeding effects of AgRP and explain its apparently subtle orexigenic action in Pomc<sup>−/−Tg/+</sup> mice. Pomc<sup>−/−</sup> mice that did not receive glucocorticoid replacement exhibited the most profound catabolic responses to icv injections of saline, but also to AgRP. These latter results support previous studies showing that AgRP’s actions on energy balance in adrenalectomized rats are glucocorticoid-dependent (26). Severe dysregulation of their HPA axis due to adrenal insufficiency (39, 41, 42) and up-regulated CRH expression in the PVH likely explain the paradoxical anorectic response of Pomc<sup>−/−</sup> mice.

Independently of the modulation of food intake, AgRP also modulates energy expenditure (43, 44). We therefore performed indirect calorimetry to determine if the weight gain in Pomc<sup>−/−Tg/+</sup> mice could be partly due to decreased VO<sub>2</sub> and/or modification in energy substrate utilization. Under specific experimental conditions where access to food was limited to 16h each day, a significant but discrete increase in food consumption together with decreased basal metabolic rate and increased average RER were measured in the mice. Compared to Pomc<sup>+/+Tg/+</sup> mice, the AgRP responses observed in Pomc<sup>−/−Tg/+</sup> mice were of smaller amplitude but longer duration with delayed onset, supporting a distinct mechanism of action of AgRP based on the genotype, which may reflect the lack of short-term antagonist action at MC3/4-R.

Specific brain targets of these metabolic actions of AgRP in Pomc<sup>−/−Tg/+</sup> mice remain to be determined. The central melanocortin system regulates the activities of both the sympathetic nervous system and the hypothalamic-pituitary-thyroid axis, which act in synergy to control thermogenesis (45). The presence of AgRP but not αMSH terminals on some TRH neurons expressing MC4-R suggests that these neurons are good candidates (16). Using MC4-R deficient mice, several laboratories have independently demonstrated a critical role for MC4-R in maintaining basal metabolic activity (46, 47). However, short-term and long-term hyperphagic actions of AgRP were still observed in MC4-R KO mice, suggesting that MC4-R is not the only receptor to mediate effects of the orexigenic peptide. Interestingly, MC3-R regulates partitioning of fuel stores into fat rather than directly...
affecting food consumption (6, 7). In our study, the increased RER after AgRP treatment suggests a switch from carbohydrate to fat stores as a source of energy and this may be mediated through a MC3-R mechanism of action. As the hypothalamic expression of MC4-R and MC3-R was unchanged in the mutant mice, we cannot distinguish between the possible involvement of either receptor to induce AgRP’s effects.

In conclusion, neuronal-specific POMC-deficient mice that lack αMSH signaling in the CNS have increased sensitivity to melanocortin agonists and respond with altered kinetics to the feeding and metabolic actions of AgRP. These data demonstrate that exogenous AgRP can modulate energy balance in the CNS independently of MC3/4-R competitive antagonism and strongly support an inverse agonist mode of action for AgRP in vivo. However, it is a possibility that long-lasting AgRP actions in Pomp+-Tg/+ mice may be relayed by a mechanism involving receptors distinct from MC3 and MC4-R. To further test the contribution of each proposed AgRP mode of action, utilization of additional pharmacological tools is necessary. If endogenous AgRP actually functions as an inverse agonist at either MC-R, a pure selective competitive MC-R antagonist should block endogenous AgRP action and decrease food intake and/or body weight in POMC-deficient mice, while having the opposite effect in wild-type mice. Current obstacles to perform or interpret the results of such experiments depend on the availability of selective compounds and the possibility that synthetic antagonists may also behave as inverse agonists. Alternatively, utilization of siRNA technology to block endogenous AgRP in POMC-deficient mice or breeding of AgRP KO to neural-specific POMC-deficient mice to create double AgRP/nPOMC KO mice would be useful approaches to better understand the mechanism of action of the endogenous orexigenic peptide.

The physiological significance of the putative inverse agonist action in humans still remains to be determined. A recent study in obese patients that identified mutations in the extracellular N terminal domain of MC4-R associated with loss of constitutive activity suggests that modulation of constitutive activity in wild-type receptors by inverse agonists could be important to maintain long-term energy homeostasis in humans (21). Supporting this possibility are recent reports that the human MC4-R undergoes ligand-independent cycles of endo- and exocytosis in transfected neuroblastoma cells and immortalized hypothalamic neurons (48) and antibodies directed against an epitope in the N terminal domain of the MC4-R act as inverse agonists in cell lines and intact animal models (49). Assessment of the effects of AgRP in knock-in mouse models containing humanized MC4-R with the inactivating N terminal mutations, or other characterized, constitutively active receptors that respond solely to a synthetic ligand (RASSLs) (50) would be useful to confirm that AgRP’s mechanism of action is partly mediated through modulation of MC4-R constitutive activity.

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FIGURE LEGENDS

Figure 1. Effects of icv injection of MTII on food intake and body weight.
(A) 2h food intake, (B) 24h food intake and (C) 24h body weight change after injection of MTII 0.5 nmol in Pomc+/+Tg/+ control, Pomc−/−Tg/+ and Pomc−/− mice at the onset of the dark cycle. Repeated measures ANOVAs showed a significant effect of 0.5 nmol MTII treatment on 2h food intake in all genotypes (F_{2,21} = 46.6, P < 0.0001) and on 24h food intake (F_{2,21} = 38.3, P < 0.0001) and nearly a significant effect on body weight change (F_{2,21} = 3.4, P = 0.08). The effects of MTII 0.5 nmol were prolonged over 24h in Pomc−/−Tg/+ and Pomc−/− mice but not in Pomc+/+Tg/+ controls. *P<0.05, **P<0.01 vs saline, paired T-test analyses. Data are means±SEM, n=7-10.

Figure 2. Feeding effects of icv injection of AgRP on the anorectic response to MTII.
(A) 2h food intake and (B) 24h food intake after administration of saline, AgRP 0.5 nmol alone, MTII 0.1 nmol alone, or co-administration of MTII 0.1 nmol and AgRP 0.5 nmol at the onset of the dark cycle in Pomc+/+Tg/+ control, Pomc−/−Tg/+ and Pomc−/− mice. Repeated measures ANOVAs showed a significant effect of the treatments on 2h food intake in all genotypes (F_{3,29} = 18.5, P<0.0001) which was not observed after 24h (F_{3,29}=1.7, P=0.17). Post-hoc analyses showed that the MTII treatment decreased 2h food intake significantly in all genotypes (P<0.0001 vs saline) and that AgRP antagonized the anorectic action of MTII (P<0.0001 MTII+AgRP vs MTII). Data are means±SEM, n=7-14, except Pomc−/−Tg/+ mice (n=3).

Figure 3. Short-term feeding effects of icv injection of AgRP and ghrelin.
(A) 2h food intake after injection of AgRP 0.5 nmol during the light cycle in Pomc+/+Tg/+ and Pomc−/−Tg/+ mice. (B) 2h food intake after injection of ghrelin 1 nmol during the light cycle in Pomc+/+Tg/+ control, Pomc−/−Tg/+ and Pomc−/− mice. Repeated measures ANOVAs showed a significant effect of 0.5 nmol AgRP (F_{1,13} = 7.7, P=0.0016) and of 1 nmol ghrelin injection (F_{2,33} = 12.3, P=0.0013). Pomc+/+Tg/+, but not Pomc−/−Tg/+ mice, responded significantly to AgRP treatment. Pomc+/+Tg/+ and Pomc−/−Tg/+ but not Pomc−/− mice, responded significantly to ghrelin treatment. *P<0.05, **P<0.0001 vs saline, paired T-test analyses. Data are means±SEM, n=6-9 for AgRP treatment, n=6-13 for ghrelin treatment.

Figure 4. Short and long-term effects of AgRP on food intake and body weight.
(A, D, G and J) 2h food intake, (B, E, H and K) daily 24h food intake and (C, F, I and L) body weight change over a period of 96h after a single injection of 0.5 nmol AgRP at the onset of the dark cycle in Pomc+/+Tg/+ control (A, B, C), Pomc−/−Tg/+ (D, E, F), Pomc−/− (G, H, I) and Pomc+/− (J, K, L) mice. Baseline corresponds to 24h food intake measured in non-injected animals. Paired T-test (saline vs AgRP) applied on individual genotypes showed a significant effect of 0.5 nmol AgRP on 24h food intake in Pomc+/+Tg/+ control and Pomc+/− mice but not in Pomc−/−Tg/+ and Pomc−/− mice and on body weight gain in Pomc+/+Tg/+ control, Pomc+/−Tg/+ and Pomc+/− but not Pomc−/− mice. *P<0.05, **P<0.01, ***P<0.001 vs saline. All data are mean±SEM, n=5-8.

Figure 5. Long-term effects of AgRP on body weight, food intake, VO_{2} and respiratory exchange ratio (RER) in mice with restricted access to food.
(A, B) Representative body weights, (C, D) average 24h food intake, (E, F) average VO_{2} and (G, H) average respiratory exchange ratio (RER) in Pomc+/+Tg/+ control and Pomc−/−Tg/+ mice.
housed for 6-8 h during the day-time in oxymax chambers without access to food and water. Each animal received saline and AgRP 0.5 nmol in a randomized manner, at the onset of the dark cycle (indicated by plain arrows) and parameters were recorded up to 168h after the injection. In addition, all animals received an injection of saline 2 days before the injection of the drug (indicated by dotted arrows). Data marked as “basal saline” were the mean values of these 2 days. Body weight data are individual values of 2 representative animals per genotype (A and B). Gray shades highlight the time-frame of the effect of AgRP on body weight (A and B), which is different in Pomer+/+Tg/+ control (3 days) and Pomer-/-Tg/+ mice (6 days). All other data are mean±SEM, n=7-8 (C-F). Paired T-tests showed a significant effect of AgRP on food intake, VO2 and RER. *P<0.05, **P<0.01, ***P<0.001 vs saline.

Figure 6. Relative quantification (RQ) of MC4-R, MC3-R, AgRP, NPY, and CART gene expression in the hypothalamus. (A) MC4-R mRNA (F2,21=0.040, P=0.961), (B) MC3-R mRNA (F2,19=1.048, P=0.3701), (C) AgRP mRNA (F2,19=13.759, P=0.0002), (D) NPY mRNA (F2,19=4.605, P=0.0234), and (E) CART mRNA (F2,19=4.516, P=0.0249) levels in Pomer-/-Tg/+ and Pomer-/- mice compared to Pomer+/+Tg/+ control mice according to the formula RQ = 2^\(\Delta\Delta C_T\). C_T was defined as the threshold cycle of PCR at which amplified product was detected and 2^\(\Delta\Delta C_T\) represents the fold change in gene expression normalized to 18S and relative to Pomer+/+Tg/+ control mice. \(\Delta\Delta C_T\) was calculated as follow : (C_T, MC4-R- CT,18S)Pomer-/-Tg/+ - (C_T, MC4-R- CT,18S)Pomer+/+Tg/+. Data are the means (horizontal bars) and scattergrams of all individual RQ (n=6-10) normalized to 18S. *P<0.05, **P<0.01, and ***P<0.0001 by Fisher’s PLSD for the indicated pair-wise comparisons.
Figure 1

A

2h Food intake (g)

Saline

MTI 0.5 nmol

+/+, Tg/+  
-/-, Tg/+  
-/-, +/+  

**

B

24h Food intake (g)

+/+, Tg/+  
-/-, Tg/+  
-/-, +/+  

ns  
**  
**

C

Delta BW (g)

+/+, Tg/+  
-/-, Tg/+  
-/-, +/+  

ns  
**  
*
Figure 2

A

2h Food intake (g)

Saline
AgRP 0.5 nmol
MTII 0.1 nmol
MTII 0.1 nmol + AgRP 0.5 nmol

+/+, Tg/+  
-/-, Tg/+  
-/-, +/-  
+/-, +/-  

B

24h Food intake (g)

+/+, Tg/+  
-/-, Tg/+  
-/-, +/-  
+/-, +/-  

AgRP actions in POMC-deficient mice
Figure 3

A

2h Food intake (g)

Saline
AgRP 0.5 nmol

+/+, Tg/+  ns

B

2h Food intake (g)

Saline
Ghrelin 1 nmol

+/+, Tg/+  ns

-/-, Tg/+  ns

-/-, ++/+
Figure 4
Figure 5
Figure 6