

A C-terminal fragment of Agouti-related protein increases feeding and antagonizes the effect of alpha-melanocyte stimulating hormone *in vivo*

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Agouti-related protein (Agrp) is present in rat and human hypothalamus and is structurally related to agouti protein. Over-expression of either of these proteins results in obesity. However the effect of exogenous Agrp and its *in vivo* interaction with alpha-melanocyte stimulating hormone (α MSH), the likely endogenous melanocortin 3 and 4 receptor (MC3-R and MC4-R) agonist, have not been demonstrated. We report that 1nmol of Agrp(83-132), a C-terminal fragment of Agrp, when administered intracerebroventricularly (ICV) into rats, increased food intake over a 24-h period (23.0 ± 1.4 g saline vs 32.9 ± 2.3 g Agrp, $p < 0.05$). The hyperphagia was similar to that seen when 1nmol of the synthetic MC3-R and MC4-R antagonist SHU9119 was given ICV (19.6 ± 1.8 g saline vs 32.5 ± 1.7 g SHU9119, $p < 0.001$). Both Agrp(83-132) and SHU9119 blocked the reduction in 1-h food intake of ICV α MSH at the beginning of the dark phase. This effect occurred independently of whether the antagonists were administered simultaneously, or nine hours prior, to the α MSH. We have also shown Agrp(83-132) is an antagonist at the MC3-R and MC4-R, with similar inhibition of cAMP activation to that previously reported for the full length peptide. In conclusion, Agrp(83-132) administered ICV increases feeding with long lasting effects and is able to inhibit the action of α MSH. This interaction may be mediated by the MC3-R and/or MC4-R.

Introduction

The ubiquitous expression of Agouti protein in the agouti (A^y) mouse causes obesity, hyperphagia and hyperinsulinaemia. It was reported in 1994 that Agouti protein was a high affinity antagonist at the melanocortin-4 receptor (MC4-R) {1}. This receptor is found exclusively within the CNS {2} and an endogenous agonist alpha-melanocyte stimulating hormone (α MSH) had previously been reported to inhibit food intake {3}. Thus it was hypothesised that agouti protein was altering food intake by antagonising hypothalamic MC4 receptors.

The importance of the MC4-R in feeding behaviour was supported by the generation of mice lacking this receptor which were found to exhibit an obesity phenotype similar to the *agouti* mouse {4}. Further evidence came from experiments with a synthetic MC3/4-R antagonist, SHU9119, which caused an increase in feeding when administered intracerebroventricularly (ICV) to mice {5}.

The physiological importance of Agouti protein, which is not normally expressed in the CNS, remained unclear until the identification of Agouti-related protein (Agrp) by two groups in 1997 {6, 7}. Agrp is a 132 amino acid peptide that is normally expressed in the rodent and human hypothalamus. Human Agrp was found to be 25% identical to human agouti protein and an antagonist at both MC3-R and MC4-R {8}. Transgenic mice overexpressing Agrp were found to be phenotypically identical to the MC4-R knock-out mice {9}.

Thus a novel hypothalamic appetite-regulation mechanism had been proposed. The MC4-R activity may be modified by both an endogenous agonist, such as α MSH, and an endogenous antagonist, such as Agrp. Although this has been suggested by *in vitro* data, our aim was to test this hypothesis *in vivo*. For these studies we

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used Agrp(83-132), a recently described C-terminal fragment of Agrp with high affinity for the MC1-R {10}.

Methods

Peptides

Agrp(83-132) was purchased from Phoenix Pharmaceuticals, Inc. (CA, USA). This fragment is made on an automated peptide synthesizer, cyclized in solution and purified by HPLC. Determination of its molecular mass confirmed a protein containing the full complement of 5 disulphide bridges {10}. SHU9119 was purchased from Peptide Products (UK). α MSH was purchased from Peninsula (UK).

Animals

Adult male Wistar rats (200-250g) were maintained in individual cages under controlled temperature (21-23°C) and light (12-h of light, 12-h of darkness, lights on at 7am). Animal procedures undertaken were all approved by the British Home Office Animals Scientific Procedures Act 1986 (Project Licence no.90/1077).

ICV cannulation and injections

Animals were implanted with permanent 22-gauge stainless steel cannulae projecting to the IIIrd ventricle and peptides were administered as previously described {11}. All compounds were injected in 10 μ l 0.9% saline.

Study 1 – Dose-response and time course of the effect of ICV Agrp(83-132) or SHU9119

A dose-response study for both compounds was performed in satiated animals during the early light phase (9 to 10 am). Animals were injected with either saline or Agrp(83-132) (0.01 to 3nmol, 7 groups, n=7-9) and saline or SHU9119 (0.1 to 2.5nmol, 5 groups, n=8-11).

Immediately after injection, animals were returned to cages containing a known amount of chow. At 1, 2, 4, 8 and 24-h after each injection remaining food was weighed to the nearest 0.1g.

Three groups of animals (n=8-13) were then studied at the beginning of the dark phase. Animals were injected with either saline, 1nmol Agrp(83-132) or 1nmol SHU9119, since these were the lowest doses above which no further increase in feeding was seen in the previous study. At 1, 2, 4 and 14-h after injection, remaining food was weighed.

Study 2 - Simultaneous ICV administration of Agrp(83-132) or SHU9119 with α MSH

Six groups of animals (n=12-14) were studied at the beginning of the dark phase. Animals were injected with either saline, 1nmol α MSH, 1nmol Agrp(83-132), 1nmol SHU9119, α MSH plus Agrp(83-132) or α MSH plus SHU9119. The α MSH dose was chosen as the lowest effective dose from a dose-response study performed at the beginning of the dark phase (data not shown). At 1, 2, 4 and 14-h after injection, remaining food was weighed.

Study 3 - ICV Administration of Agrp(83-132) or SHU9119 nine hours prior to α MSH

Animals were divided into four groups (n=17-19): saline-saline, saline- α MSH, Agrp(83-132)-saline or Agrp(83-132)- α MSH. In early light phase animals were injected with either saline or 1nmol Agrp(83-132). Nine hours later, at the beginning of the dark phase, animals were injected with either saline or 1nmol α MSH. The remaining food was measured before the dark phase injection and at 1, 2, 4 and 14-h after the second injection. This study was repeated with 1nmol SHU9119 replacing the Agrp.

Receptor Antagonist Studies

Intracellular cAMP concentrations were measured with a scintillation proximity assay using anti-cAMP antibody and 125 I-cAMP (Amersham). HEK 293 cells expressing the human MC-3R and MC-4R were incubated in DMEM containing 10% FBS, 500 μ g/ml G418 and 0.1nM isobutylmethylxanthine with Agrp(83-132) (concentration 0.003 to 200nM) in the presence of 20nM [Nle⁴D-Phe⁷]- α MSH for 1-h at room temperature. Incubations were terminated by removal of culture medium and the addition of 60 μ l of 70% ethanol. After 30-min extraction, 10 μ l of the ethanol extract was assayed in the cAMP assay.

Statistical analysis

Food intake (g) is expressed as means \pm SEM. All results obtained from feeding studies were analysed by ANOVA followed by Bonferroni post-hoc analysis. Values of $p < 0.05$ are considered significant.

Results

Study 1 - Dose-response and time course of the effect of ICV Agrp(83-132) or SHU9119

Both compounds increased feeding in a dose-dependent manner being most significant at 24-h post injection

(ANOVA of $p < 0.001$ for both studies, data not shown). The lowest fully effective dose was 1nmol of Agrp(83-132) and 1nmol SHU9119 with a 24-h food intake of 23.0 \pm 1.4 g saline vs 32.9 \pm 2.3 g Agrp(83-132), $p < 0.05$ and 19.6 \pm 1.8 g saline vs 32.5 \pm 1.7 g SHU9119, $p < 0.001$ (fig 1a&b). The effect on food intake seen had a similar time course and potency for both antagonists. At 24-h post injection 1nmol of Agrp(83-132) stimulated feeding to 143% of the saline group and 1nmol SHU9119 stimulated feeding to 184% of the saline group.

When given at the beginning of the dark phase both compounds increased feeding but these effects were statistically significant only at 14-h post injection (16.1 \pm 1.2 g saline, 26.7 \pm 2.5 g Agrp(83-132), 24.8 \pm 1.7 g SHU9119; $p < 0.005$ for both antagonists vs saline group, data not shown).

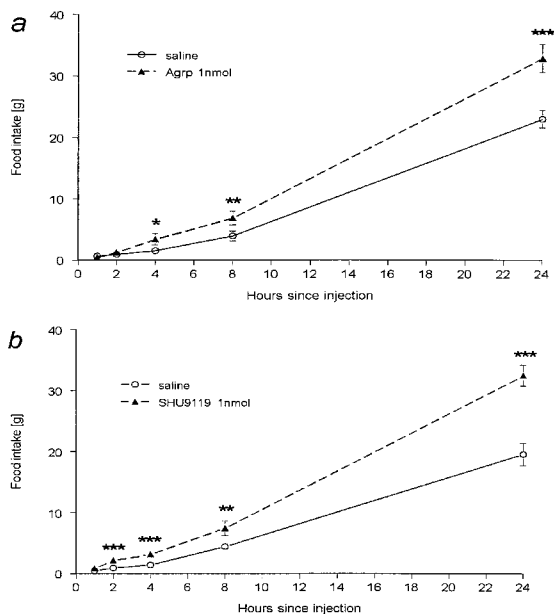


Figure 1: Effect of a single ICV injection of peptide or saline given in early light phase on food intake over a 24-h period. a, 1nmol Agrp(83-132). b, 1nmol SHU9119. Significance values for individual time points are indicated. *= $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$ vs. saline.

Study 2 - Simultaneous ICV administration of Agrp(83-132) or SHU9119 with α MSH

At 1-h after injection, α MSH significantly decreased feeding (0.6 \pm 0.2 g α MSH vs 1.9 \pm 0.2 g saline, $p < 0.01$, fig 2a&b). Agrp(83-132) or SHU9119 alone did not significantly increase feeding (1.6 \pm 0.3 g Agrp, 2.5 \pm 0.3 g SHU9119, $p = ns$ vs saline). Both Agrp(83-132) and SHU9119 blocked the reduction in feeding seen with α MSH at 1-h. One nmol of Agrp(83-132) completely blocked the α MSH effect, (1.8 \pm 0.2 g Agrp+ α MSH, $p < 0.01$ vs α MSH group). One nmol of SHU9119 attenuated the α MSH effect but this was not completely

reversed (1.6 ± 0.2 g SHU9119+ α MSH, p =ns vs α MSH group). There was no significant difference between SHU9119 and SHU9119+ α MSH group or Agrp(83-132) and Agrp(83-132)+ α MSH group. The reduction in feeding caused by the α MSH was no longer significant at 2-h post injection (data not shown).

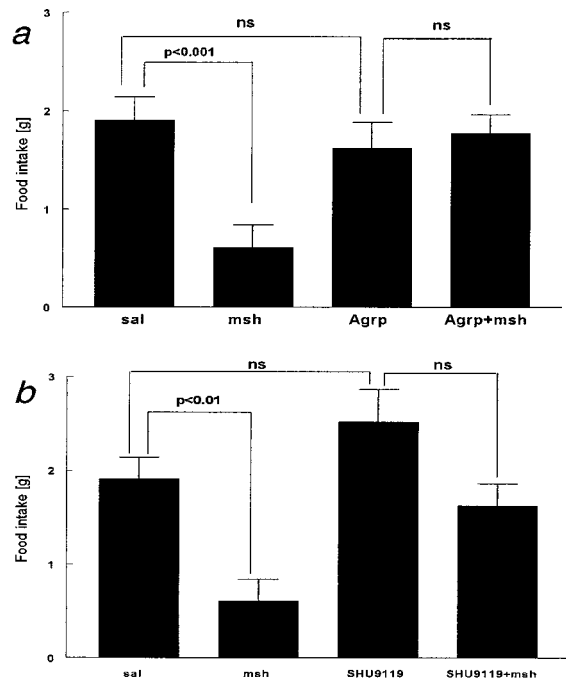


Figure 2: 1-h food intake at the beginning of the dark phase. *a*, after ICV saline, 1nmol α MSH, 1nmol Agrp(83-132) or 1nmol α MSH + 1nmol Agrp(83-132). *b*, after ICV saline, 1nmol α MSH, 1nmol SHU9119 or 1nmol α MSH + 1nmol SHU9119.

Study 3 - ICV Administration of Agrp(83-132) or SHU9119 nine hours prior to α MSH

Again, at 1-h after injection, α MSH significantly decreased feeding (0.4 ± 0.1 g saline+ α MSH vs 1.6 ± 0.3 g saline+saline, $p < 0.01$, fig 3a and 0.2 ± 0.1 g saline+ α MSH vs 1.0 ± 0.2 g saline+saline, $p < 0.05$, fig 3b). During this 1-h period Agrp(83-132) or SHU9119 alone did not significantly increase feeding (2.4 ± 0.3 g Agrp+sal, 0.9 ± 0.1 g SHU9119+sal, p =ns vs saline). Both Agrp(83-132) and SHU9119 blocked the reduction in feeding seen with α MSH at 1-h (1.6 ± 0.2 g Agrp+ α MSH, $p < 0.01$ vs saline+ α MSH group; 0.9 ± 0.1 g SHU9119+ α MSH, $p < 0.001$ vs saline+ α MSH group). There was no statistically significant difference between Agrp+saline or SHU9119+saline and their respective saline+saline groups.

Receptor Antagonist Studies

Agrp(83-132) at a concentration up to 1μ M had no effect on hMC3-R or hMC4-R activation. Agrp(83-132) antagonised 20nM NDP- α MSH induced cAMP activation with an IC_{50} of 3.4 ± 0.25 nM for hMC3-R ($n=5$) and

12.8 ± 0.37 nM ($n=5$) for hMC4-R. This was comparable to an IC_{50} of 1.0 and 3.2nM respectively previously reported for full length Agrp {8}.

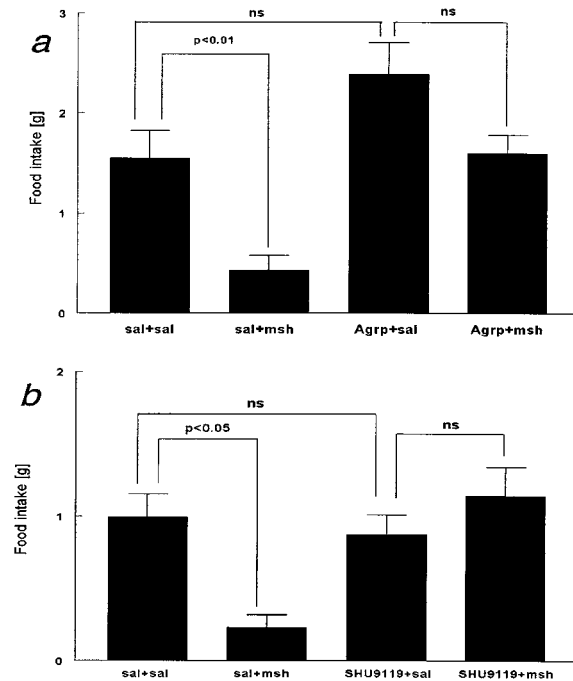


Figure 3: 1-h food intake at the beginning of the dark phase. *a*, Animals received ICV saline or 1nmol Agrp(83-132) at the beginning of the light phase followed by saline or 1nmol α MSH at the beginning of the dark phase. *b*, Animals received ICV saline or 1nmol SHU9119 in early light phase followed by saline or 1nmol α MSH at the beginning of the dark phase.

Discussion

We have shown for the first time that a 83-132 c-terminal fragment of Agrp causes an increase in feeding when administered ICV to rats. Both Agrp(83-132) and the synthetic MC3/4-R antagonist SHU9119 can block the reduction in feeding seen with the endogenous MC-R agonist α MSH, at a time when no increase in feeding was observed when the antagonists were administered alone. Furthermore, this effect was still observed nine hours after ICV administration of antagonist. We have also demonstrated that Agrp(83-132) has antagonist activity at MC3-R and MC4-R *in vitro*. These data provide evidence that the novel protein Agrp may control feeding by antagonism of the effects of α MSH probably through one of the melanocortin receptors.

The fact that the Agrp is still able to block the effect of α MSH nine hours after administration suggests that persistent antagonism of the MC3/4-R is an important mode of action. Agrp and SHU9119 may both bind tightly to the receptor and act as irreversible antagonists or cause down-regulation or desensitisation of the receptor. This is seen *in vitro* at the MC4-R, but not the MC3-R, where

increasing doses of *Agrp* seem to reduce maximal stimulation of cAMP accumulation by α MSH, rather than causing a right-shift in the dose response curve {7}.

A receptor system having both an endogenous agonist and antagonist appears unique, with no parallel previously described in neuroendocrinology. The endogenous agonist is likely to be one or more of the POMC products produced in the arcuate nucleus of the hypothalamus. POMC processing here is generally agreed to be similar to that seen in the *pars intermedia* rather than that in the anterior pituitary {12} although active (amidated and N-acetylated) α MSH forms only a small proportion {13}. Alpha-MSH immunostaining is found in areas of rat brain known to express MC3/4-R and is therefore a good candidate for the endogenous agonist {14}.

It therefore appears that both the C-terminal fragment of *Agrp* and agouti protein retain their biological activity {15}. This cysteine-rich region is the area of highest homology between the two proteins. Recently the agouti residues important for melanocortin receptor binding were identified {16}. Interestingly all these amino acids are conserved within *Agrp*.

The long-lasting effects of the *Agrp* are notable. Unlike other neuropeptides, such as neuropeptide Y, galanin and melanin concentrating hormone, which stimulate a rapid but short lived increase in food intake following ICV injection {11, 17, 18}, *Agrp* appears to stimulate a small but long lasting increase persisting for at least 24 hours. Interestingly, the feeding inhibitory actions of leptin follow a similar time course {19}. Just as the interactions of leptin with numerous other neuropeptides have now been established, this may prove to be the case for *Agrp* {20, 21, 22, 23}. Indeed, it has already been reported that the MC4-R system is downstream of leptin's effects in reducing food intake {24}.

These studies provide further direct evidence of an *in vivo* interaction between *Agrp* and α MSH and emphasise the involvement of this unique receptor system in the control of food intake. It will be of value to determine exactly what role *Agrp*, the POMC products, MC3-R and MC4-R have and how they fit into the now complex hypothalamic feeding circuit.

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