# γ-MSH increases intracellular cAMP accumulation and GnRH release in vitro and LH release in vivo

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Received 31 January 2003; revised 19 March 2003; accepted 1 April 2003

First published online 28 April 2003

Edited by Veli-Pekka Lehto

**Abstract The roles of the melanocortin 3 receptor (MC3-R) and its agonist, γ2-melanocyte-stimulating hormone (γ2-MSH) in the regulation of the hypothalamo-pituitary-gonadal (HPG) axis are poorly understood. Here we show γ2-MSH stimulated intracellular cAMP accumulation and gonadotrophin-releasing hormone (GnRH) secretion in the immortalised GnRH cell line GT1-7. The MC3/4-R antagonist Agrp blocked these actions. Reverse transcriptase polymerase chain reaction demonstrated GT1-7 cells express MC3-R mRNA. γ2-MSH also stimulated GnRH release from hypothalamic explants. In vivo, γ2-MSH administration into the medial preoptic area significantly increased plasma luteinising hormone. MC3-R and γ2-MSH may modulate the HPG axis.**


*Key words:* Melanocortin; Gamma melanocyte-stimulating hormone; Alpha melanocyte-stimulating hormone; Gonadotropin-releasing hormone; Hypothalamus

## 1. Introduction

The hypothalamic melanocortin system comprises the melanocortin 3 and 4 receptors (MC3-R and MC4-R), the endogenous MC3/4-R antagonist, agouti-related protein (Agrp) and the agonists α- and γ-melanocyte-stimulating hormones (α-MSH and γ-MSH). α-MSH activates MC3-R and MC4-R equally but γ2-MSH has a higher affinity for MC3-R [1,2]. Whilst MC4-R may modulate energy homeostasis, the role of MC3-R remains unclear.

Anatomical evidence suggests the melanocortin system may influence reproductive function. MC4-R and MC3-R mRNA [1,3] and α-MSH [4] and γ-MSH immunoreactivity are found in the medial preoptic nucleus (MPO), an area rich in gonadotrophin-releasing hormone (GnRH) neurones. Pro-opiomelanocortin (POMC) neurones synapse directly onto GnRH neurones [5] and the POMC products, α-MSH and β-endorphin have been demonstrated to influence gonadotrophin release (see [6] for review). Arcuate Agrp-containing neurones also project to the MPO [7]. This suggests GnRH neurones may be influenced by the melanocortin system.

The aims of these studies were to (a) determine whether α-MSH and γ2-MSH act directly on GnRH neurones; (b) identify the melanocortin receptor(s) resulting in GnRH release; (c) examine the effects of α-MSH and γ2-MSH on GnRH release from hypothalamic explants; and (d) confirm these findings in vivo by measurement of plasma gonadotrophins following MPO injection of α-MSH and γ2-MSH in male rats.

## 2. Materials and methods

### 2.1. Peptides

[Nle$^4$,D-Phe$^7$]-α-MSH (NDP-MSH) (a long-acting synthetic analogue of α-MSH), α-MSH and γ2-MSH were purchased from Bachem Laboratory plc (St. Helens, Merseyside, UK). Agrp (83–132) was purchased from Phoenix Pharmaceuticals (Belmont, CA, USA). Other reagents were purchased from BDH (Poole, Dorset, UK), Gibco (Renfrewshire, UK) and Sigma (Poole, Dorset, UK).

### 2.2. Animals

Male Wistar rats (specific pathogen-free, Imperial College, London, UK) weighing 250–300 g were individually housed under controlled temperature (21–23°C) and light (12 h light, 12 h dark) with ad libitum access to food (RM1 diet, SDS UK Ltd, Witham, UK) and water. Animal procedures were approved by the British Home Office Animals Scientific Procedures Act 1986 (project license 90/1077).

### 2.3. Cell culture

The immortalised murine hypothalamic GnRH neuronal cell line GT1-7 (donated by Dr P. Mellon, University of California) [8] was cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco) as previously described (passage number 26–28) [9]. Cells were cultured ($2.5\times10^5$ per well, determined by haemocytometer counts) in 48 well plates (Nunc International, Roskilde, Denmark) in DMEM and maintained for 48 h to reach 90–95% confluence.

Briefly, for cAMP determination, cells were preincubated in serum-free DMEM containing 3-isobutyl-1-methyl-xanthine (IBMX) (2 mM) (Sigma). The medium was replaced with DMEM and IBMX either alone, with the test substance(s) or with forskolin (100 μM). After 15 min, the cells were washed and extracted in ethanol. The samples were dried and the cAMP concentration determined by a cAMP scintillation proximity assay (Amersham, UK). To assess GnRH release, cells were preincubated for 2 h in DMEM with 0.1% bovine serum albumin (Sigma). The medium was replaced with DMEM either alone, containing the test substance(s) or with KCl (59 mM) and incubated for 1 h. The GnRH concentration in the medium was determined by radioimmunoassay (RIA). All studies were repeated on at least four occasions.

### 2.4. Melanocortin receptor expression in GnRH clonal cell lines

Total RNA was isolated from GT1-7 and mouse hypothalami ($n=6$) using Trizol reagent (Gibco, Paisley, Renfrewshire, UK) according to the manufacturer's instructions. Twenty micrograms were reverse-transcribed using avian myoblastoma virus reverse transcrip-

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0014-5793/03/$22.00 © 2003 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/S0014-5793(03)00402-2

tase (RT) (Promega, Southampton, UK) in a reaction primed using oligo(dT). Half of this reaction was subjected to polymerase chain reaction (PCR) using primers obtained from the published sequence (mouse MC3-R: X74983, mouse MC4-R: AF201662). Primers were synthesised by Oswel DNA services (Southampton, UK). The primers used in the reaction for MC4-R were atactaggtagagacctgc and aaggctctgatttctgctag and for MC3-R were cgtttctctctggagaaact and ggatgttttccatcagactg. PCR for mouse GAPDH was performed at the same time as a control (primers: ctgagtatgtcgtggagtct and tgagcttcccgttctgggca). The following concentrations were used in the PCR mixture: RT mixture 10 μl, Taq DNA polymerase buffer 10 μl (Sigma), dNTP 2 μl, 5′ primer 1 μl, 3′ primer 1 μl, Taq DNA polymerase 5 U (Sigma) and glass-distilled water 75 μl. The conditions used for the PCR were 95°C for 45 s, 58°C for 30 s and 72°C for 60 s for 30 cycles. Following PCR, 1/50 of the reaction was subjected to a second round of PCR using internal primers for MC4-R (agaatttatcactcaggcac and gaaagaacagggttgactc) and for MC3-R (atctagactggacagcatcc and acgatgcccagtgccaggaa). Using these primers, an amplified fragment of 295 kb for MC4-R and 265 kb for MC3-R would be expected. Controls treated in an identical way, but with no RT added to the reverse transcription, were conducted at the same time. All the PCR reactions were analysed on 1% agarose gels.

### 2.5. GnRH release from hypothalamic explants

Hypothalami, 1.7 mm thick, were taken parallel to the base of the brain to include the MPOs. These were maintained in a static incubation system with artificial cerebrospinal fluid (aCSF) equilibrated with 95% $O_2$ and 5% $CO_2$ as previously described [10]. Following a 2 h equilibration period, the hypothalami were incubated for three sequential 45 min periods in (a) 500 μl aCSF (basal), (b) 500 μl test peptide and (c) 500 μl 56 mM KCl. Explants not showing release above basal levels following KCl were excluded. At the end of each period, the aCSF was removed and frozen at −20°C until measurement of GnRH by RIA.

### 2.6. MPO cannulation and injection

Permanent 26-gauge medial preoptic cannulae were implanted as previously described [11]. Following recovery, the animals were handled daily and habituated to experimental procedures. All peptides were dissolved in saline and the injection volume was 0.5 μl. Correct cannula placement was confirmed histologically. Animals with incorrect cannula placement were excluded from the analysis.

The feeding effects of α-MSH and $\gamma_2$-MSH were used to determine the dose of $\gamma_2$-MSH without significant feeding effects, and therefore without significant MC4-R activation, as the appropriate dose for study of gonadotrophin release. The selected doses of $\gamma_2$-MSH were 10-fold greater than those of α-MSH based on the relative bindings of α-MSH and $\gamma_2$-MSH at MC3-R. Groups of fasted rats ($n = 10$–11) were injected with saline, α-MSH (0.1 or 0.3 nmol) or $\gamma_2$-MSH (1 or 3 nmol). Food intake was measured at 1, 4 and 24 h after injection.

Groups of fed rats ($n = 10$–11) were injected with saline, α-MSH (0.3 nmol) or $\gamma_2$-MSH (3 nmol) in the early light phase and killed 20 min following injection. Blood was collected into plastic lithium heparin tubes containing 0.6 mg of apoprotinin. Plasma was separated by centrifugation and stored at −20°C. Plasma luteinising hormone (LH) and follicle-stimulating hormone (FSH) were measured by RIA.

### 2.7. RIA

GnRH levels were measured using reagents and methods kindly provided by H.M. Fraser, Medical Research Centre Reproductive Biology Unit, Edinburgh, UK as previously described [12]. Plasma LH and FSH levels were assayed using reagents and methods provided by the NIDDK and the National Hormone and Pituitary Program (Dr A. Parlow, Harbor University of California, Los Angeles Medical Center, Los Angeles, CA, USA) as previously described [13].

### 2.8. Statistical analysis

Results are shown as mean values ± S.E.M. Data from clonal cell line cAMP studies and secretion experiments are expressed as a percentage of basal release. Data from hypothalamic explant release experiments were compared by paired *t*-test between the basal period and test period. Data from cAMP, secretion and in vivo studies were compared by analysis of variance with post-hoc Dunnett's test (Systat, Evanston, IL, USA) between control and experimental groups. In all cases, $P < 0.05$ was considered to be statistically significant.

## 3. Results

### 3.1. Study 1 – cAMP accumulation in $GT_1$-7 cells

NDP-MSH dose-dependently increased intracellular cAMP concentration (Fig. 1a) (cAMP (% basal): basal 100 ± 9%, NDP-MSH (1000 nM) 3131 ± 727%, $P < 0.005$). $\gamma_2$-MSH also resulted in a dose-dependent increase in intracellular cAMP concentration (cAMP (% basal): basal 100 ± 10%, $\gamma_2$-MSH (10 μM) 1398 ± 403%, $P < 0.05$) (Fig. 1b). Agrp (100 nM) alone did not alter cAMP accumulation but Agrp 100 nM (100 × $K_i$ for the MC3/4-R) blocked NDP-MSH and $\gamma_2$-

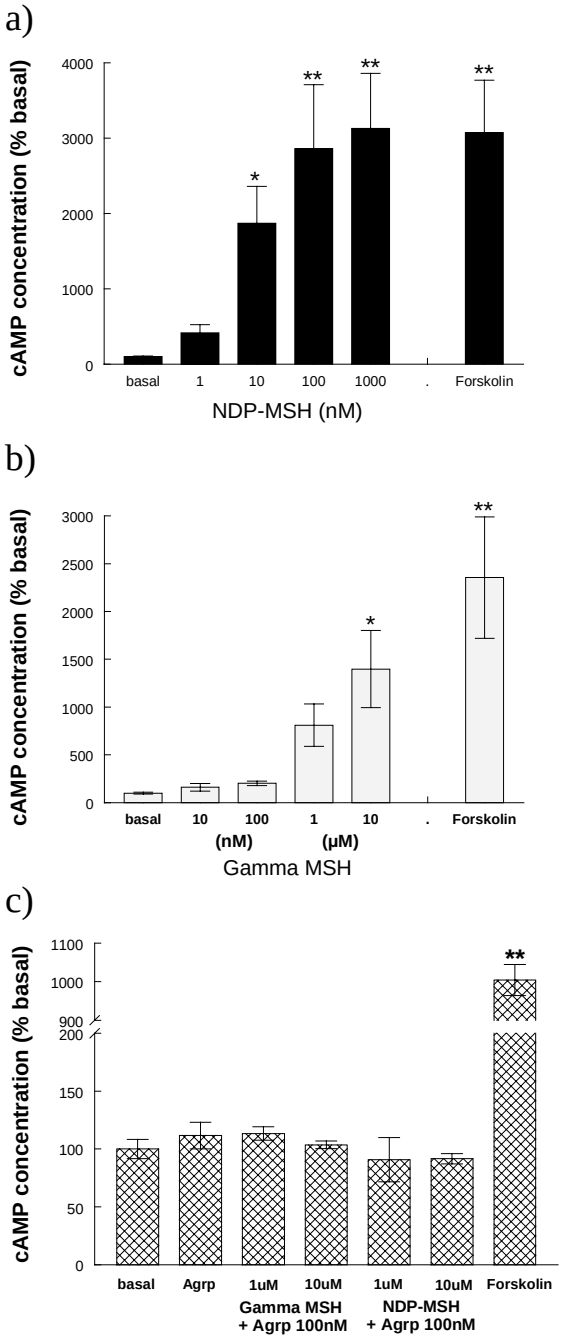


Fig. 1. The effect of (a) NDP-MSH, (b) $\gamma_2$-MSH and (c) Agrp with NDP-MSH or $\gamma_2$-MSH on intracellular cAMP concentrations in $GT_1$-7 cells. *$P < 0.05$ v. basal, **$P < 0.01$ v. basal.

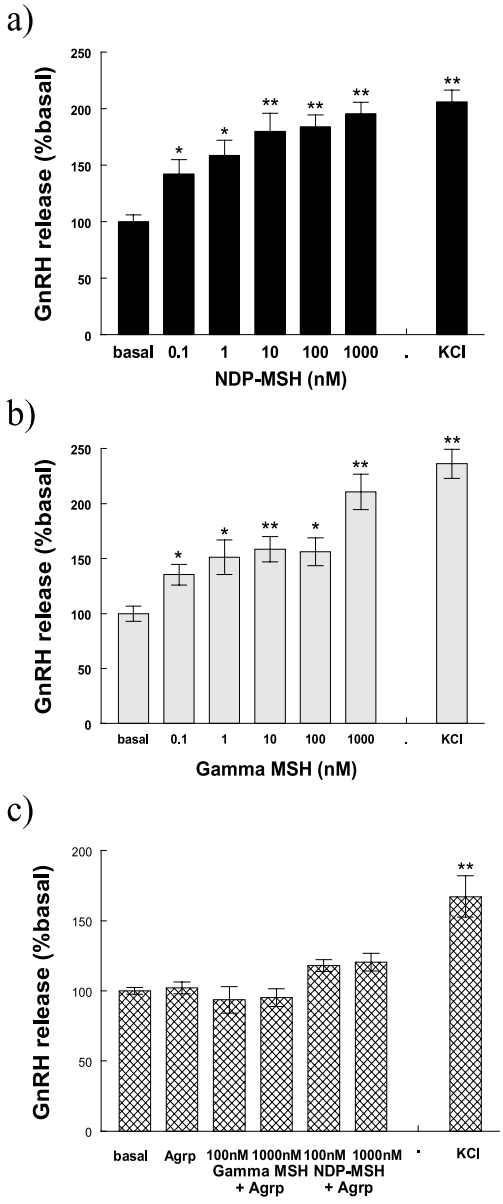


Fig. 2. The effect of (a) NDP-MSH, (b) $\gamma_2$-MSH and (c) Agrp with NDP-MSH or $\gamma_2$-MSH on GnRH release from $GT_1$-7 cells. *$P < 0.05$ v. basal, **$P < 0.01$ v. basal.

MSH cAMP accumulation (cAMP (% basal): basal 100 ± 7%, Agrp (100 nM)+$\gamma_2$-MSH (10 μM) 94 ± 13%, Agrp (100 nM)+NDP-MSH (10 μM) 132 ± 30%, $P$ = NS) (Fig. 1c).

### 3.2. Study 2 – GnRH release from $GT_1$-7 cells

NDP-MSH dose-dependently increased GnRH secretion (Fig. 2a) (GnRH release (% basal): basal 100 ± 5.9%, NDP-MSH (1000 nM) 195 ± 10%, $P < 0.01$). There was a dose-dependent rise in GnRH release in response to $\gamma_2$-MSH (GnRH release (% basal): basal 100 ± 6.9%, $\gamma_2$-MSH (1000 nM) 210 ± 15%, $P < 0.01$) (Fig. 2b). Agrp alone did not alter GnRH release; however, Agrp (100 nM) blocked $\gamma_2$-MSH and NDP-MSH stimulated GnRH release (GnRH release (% basal): basal 100 ± 2%, Agrp (100 nM)+$\gamma_2$-MSH (1000 nM) 95 ± 6%, Agrp (100 nM)+NDP-MSH (1000 nM) 120 ± 6%, $P$ = NS) (Fig. 2c).

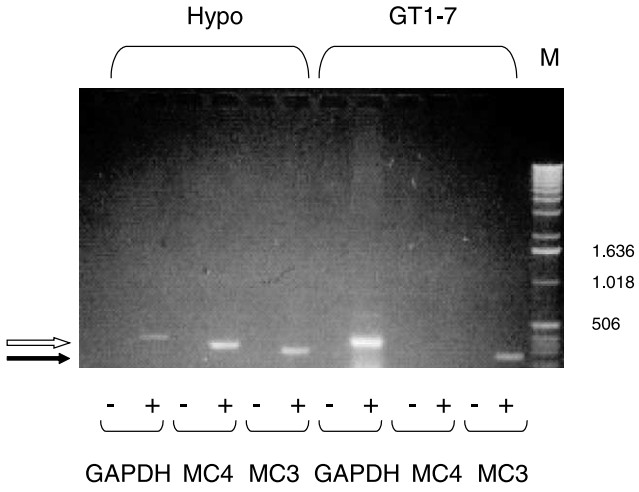


Fig. 3. Melanocortin receptor expression in $GT_1$-7 cells. Gel demonstrating PCR products following amplification with MC3-R- and MC4-R-specific primers of $GT_1$-7 cells and murine hypothalamic RNA with GAPDH as control. M, marker; →, expected size of MC3-R; ⇒, expected size of MC4-R.

### 3.3. Study 3 – melanocortin receptor expression in $GT_1$-7 cells

RT-PCR amplification of $GT_1$-7 cell RNA showed no evidence of MC4-R expression. A band of the appropriate size was visible following PCR using MC3-R-specific primers in

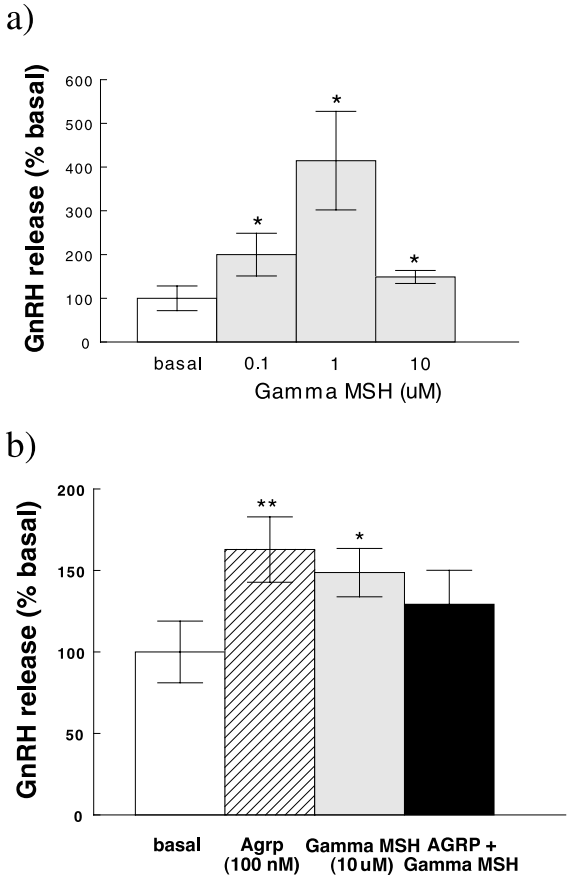


Fig. 4. The effect of (a) $\gamma_2$-MSH and (b) Agrp with $\gamma_2$-MSH on GnRH release from hypothalamic explants. *$P < 0.05$ v. basal release.

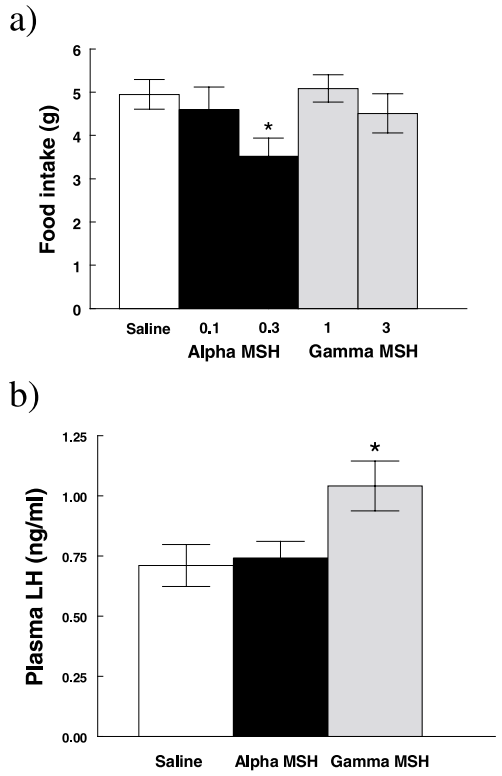


Fig. 5. Effect of saline, $\gamma_2$-MSH (3 nmol) or α-MSH (0.3 nmol) injection into the MPO on (a) food intake and (b) plasma LH. *$P < 0.05$ v. saline.

this cell line (Fig. 3). Murine hypothalamic RNA demonstrated both MC4-R and MC3-R expression.

### 3.4. Study 4 – GnRH release from hypothalamic explants

$\gamma_2$-MSH significantly increased hypothalamic GnRH release, with greatest effect at 1 μM (GnRH release (% basal): basal 100 ± 29%, $\gamma_2$-MSH (1 μM) 414 ± 112%, $P < 0.05$) (Fig. 4a). As demonstrated previously [10], Agrp significantly increased GnRH release (GnRH release (% basal): basal 100 ± 19%, Agrp (100 nM) 163 ± 20%, $P < 0.01$ ($n = 15$)). Agrp blocked $\gamma_2$-MSH-stimulated GnRH release (GnRH release (% basal): basal 100 ± 28%, Agrp (100 nM)+$\gamma_2$-MSH (10 μM) 129 ± 20%, $P$ = NS) (Fig. 4b).

### 3.5. Study 5 – plasma gonadotrophins following MPO injection

MPO injection of α-MSH (0.3 nmol) significantly reduced 1 h food intake but $\gamma_2$-MSH (3 nmol) had no significant effect (1 h food intake: saline 5.0 ± 0.3 g, $\gamma_2$-MSH (3 nmol) 4.4 ± 0.4 g, $P$ = NS, α-MSH (0.3 nmol) 3.5 ± 0.4 g, $P < 0.05$). There was no effect on food intake at later time points. Melanocortin actions on appetite are MC4-R-mediated and thus 3 nmol $\gamma_2$-MSH, which does not significantly affect food intake, was chosen to investigate the effects of MC3-R activation on gonadotrophin release.

MPO administration of $\gamma_2$-MSH (3 nmol) significantly increased plasma LH (plasma LH: saline 0.7 ± 0.1 ng/ml, $\gamma_2$-MSH (3 nmol) 1.0 ± 0.1 ng/ml, $P < 0.05$) (Fig. 5). MPO $\gamma_2$-MSH had no effect on plasma FSH (plasma FSH: saline 6.2 ± 0.4 ng/ml, $\gamma_2$-MSH (3 nmol) 6.4 ± 0.4 ng/ml, $P$ = NS). α-MSH (0.3 nmol) had no effect on plasma LH or FSH (plasma LH: saline 0.7 ± 0.1 ng/ml, α-MSH (0.3 nmol) 0.74 ± 0.1 ng/ml, $P$ = NS; plasma FSH: saline 6.2 ± 0.4 ng/ml, α-MSH (0.3 nmol) 6.4 ± 0.3 ng/ml, $P$ = NS).

## 4. Discussion

The role of the hypothalamic melanocortin system in the regulation of reproductive function is poorly understood. The above findings suggest γ-MSH and MC3-R may play a role.

Both NDP-MSH (MC3/4-R agonist) and $\gamma_2$-MSH (an agonist with greater selectivity for MC3-R) stimulate cAMP accumulation and GnRH secretion in the GnRH clonal cell line $GT_1$-7. The MC3/4-R antagonist Agrp blocks these actions. Thus both MC3-R and MC4-R agonists act directly on GnRH cells. This is supported by anatomical evidence demonstrating POMC neurones to synapse directly onto GnRH neurones [14].

Increases in intracellular cAMP are seen at higher peptide concentrations than those which increase GnRH release. A small local rise in cAMP may significantly increase GnRH release but would not significantly increase whole cell cAMP. However, low concentrations of $\gamma_2$-MSH may activate an additional second messenger system to elicit secretion, such as has been postulated in the mammolactotroph cell line $GH_3$ [15]. In $GH_3$ cells, γ-MSH increases intracellular calcium concentrations in the absence of known melanocortin receptor mRNA. The MC3/4-R antagonist SHU9119 does not block this effect. In the $GT_1$-7 cells used here, MC3-R mRNA was detected and Agrp blocked the effect of $\gamma_2$-MSH on GnRH release but this does not exclude the possibility of an additional novel $\gamma_2$-MSH receptor.

In this study, MC3-R but not MC4-R mRNA was detected in the $GT_1$-7 cells. However, Dumont et al. [16] and Khong et al. [17] have reported detectable MC4-R expression in $GT_1$-7 cells, along with MC3-R expression in the latter study. There have been conflicting reports for other receptors expressed in $GT_1$-7 cells such as oestradiol [18,19] and the γ-aminobutyric acid receptor subtypes [20,21]. Relative receptor expression levels in $GT_1$-7 cells may be influenced by different clonal expansion or by environment [22]. It is possible low levels of MC4-R expression were missed in our study, though it was repeated on several occasions; however, such low levels of MC4-R are unlikely to be of biological significance and it is therefore likely our results are the consequence of actions via MC3-R. Our findings extend previous studies by demonstrating that not only is MC3-R expressed in $GT_1$-7 cells, but also that it may mediate the effects of melanocortin agonists on GnRH release. It would be interesting to confirm these findings in $GT_1$-7 cells over-expressing MC3-R.

The effects of $\gamma_2$-MSH on GnRH release from hypothalamic explants are similar to those shown in $GT_1$-7 cells. Hypothalamic GnRH release is significantly increased by $\gamma_2$-MSH and Agrp blocks this effect. However, GnRH release is attenuated in response to high concentrations of $\gamma_2$-MSH resulting in a bell-shaped dose–response curve.

The effects of α-MSH and $\gamma_2$-MSH on gonadotrophin release were examined in vivo. Melanocortin feeding effects are thought to be mediated via MC4-R [2]. γ2-MSH (3 nmol) did not significantly reduce food intake and probably does not significantly bind MC4-R. At this dose, $\gamma_2$-MSH significantly increased plasma LH but α-MSH had no effect. This would suggest the effects of $\gamma_2$-MSH on LH are mediated by MC3-R. There was no effect of MPO area administration of $\gamma_2$-MSH

on plasma FSH. Electrophysiological studies have suggested pituitary FSH release is regulated by the anterior hypothalamic area (AHA) rather than the MPO area [23]. It would be of interest to determine the effect of $\gamma_2$-MSH injection into the AHA on plasma FSH.

Several previous studies have examined the effect of α-MSH on gonadotrophin release in vivo. Scimonelli et al. [24] demonstrated α-MSH to suppress LH whilst Khorram et al. [25] showed no effect of α-MSH in ovariectomised females and Newman et al. [26] reported a stimulation of LH due to α-MSH. Thus, the LH response to α-MSH appears to differ with administration site and steroid environment. We have demonstrated that Agrp stimulates GnRH release from hypothalamic explants and also previously shown it increases LH release in vivo [10]. This would suggest an inhibitory melanocortin tone which might be mediated by α-MSH. Since Agrp has no effect alone on GnRH release from $GT_1$-7 cells and blocks the stimulatory effect of α-MSH and $\gamma_2$-MSH, this stimulatory effect of Agrp is unlikely to be directly at the level of the GnRH neurone but might be on an inhibitory interneurone.

Rodent and human MC3-R or MC4-R null mutations have normal fertility and plasma gonadotrophins [27,28]. Developmental compensatory mechanisms may mask the roles of MC3-R and MC4-R in the hypothalamo-pituitary-gonadal (HPG) axis. However, there has been no dynamic testing of the HPG axis in these mice or humans and subtle defects may not be detected on baseline measurements alone. In contrast, ubiquitous ectopic expression of the MC4-R antagonist Agouti in Agouti yellow mice ($A^y$) results in reduced fertility late in life [29]. Further dynamic examination of the HPG axis in mice lacking melanocortin receptors or with over-expression of melanocortin antagonists would be of interest.

Our studies suggest that $\gamma_2$-MSH stimulates GnRH release directly from GnRH neurones, increases hypothalamic GnRH release and gonadotrophin release in vivo. This may suggest a role for MC3-R in regulation of the HPG axis. Further work is needed to elucidate the roles of MC3-R and MC4-R in the HPG axis and there is a particular need for highly selective melanocortin receptor agonists and antagonists.

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