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Melanin-concentrating hormone stimulates human growth hormone secretion: a novel effect of MCH on the hypothalamic-pituitary axis

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Segal-Lieberman, Gabriella, Hadara Rubinfeld, Moran Glick, Noga Kronfeld-Schor, and Ilan Shimon. Melanin-concentrating hormone stimulates human growth hormone secretion: a novel effect of MCH on the hypothalamic-pituitary axis. *Am J Physiol Endocrinol Metab* 290: E982–E988, 2006; doi:10.1152/ajpendo.00138.2005.—Melanin-concentrating hormone (MCH), a 19-amino acid orexigenic (appetite-stimulating) hypothalamic peptide, is an important regulator of energy homeostasis. It is cleaved from its precursor prepro-MCH (ppMCH) along with several other neuropeptides whose roles are not fully defined. Because pituitary hormones such as growth hormone (GH), ACTH, and thyroid-stimulating hormone affect body weight and composition, appetite, insulin sensitivity, and lipoprotein metabolism, we investigated whether MCH exerts direct effects on the human pituitary to regulate energy balance using dispersed human fetal pituitaries (21–22 wk gestation) and cultured GH-secreting adenomas. We found that MCH receptor-1 (MCH-R1), but not MCH receptor-2, is expressed in both normal (fetal and adult) human pituitary tissues and in GH cell adenomas. MCH (10 nM) stimulated GH release from human fetal pituitary cultures by up to 62% during a 4-h incubation ($P < 0.05$). Interestingly, neuropeptide EI (10 nM), which is also cleaved from ppMCH, increased human GH secretion by up to 124% in fetal pituitaries. A milder, albeit significant, induction of GH secretion by MCH (20%) was seen in cultured GH-secreting pituitary adenomas. A comparable stimulation of GH secretion was seen when cultured mouse pituitary cells were treated with MCH. Treatment of cultured GH adenoma cells with MCH (100 nM) induced extracellular signal-regulated kinases 1 and 2 phosphorylation, suggesting activation of MCH-R1. In aggregate, these data suggest that MCH may regulate pituitary GH secretion and imply a potential cross-talk mechanism between appetite-regulating neuropeptides and pituitary hormones.

prepro-melanin-concentrating hormone; adrenocorticotrophic hormone; thyroid-stimulating hormone

MELANIN-CONCENTRATING HORMONE (MCH) is a 19-amino acid hypothalamic peptide and an important regulator of energy balance. It is an orexigenic agent in mammals, acutely stimulating feeding when injected in rat brains (29, 31, 42). Mice lacking MCH or its receptor are lean and have an increased metabolic rate (23, 38), whereas mice overexpressing MCH are mildly obese and have an increased susceptibility to diet-induced obesity (22).

The synthesis of MCH occurs primarily in the lateral hypothalamus and zona incerta regions of the brain (25, 26, 39). It is cleaved from its precursor prepro-MCH (ppMCH) by prohormone convertases along with the following two other peptides: neuropeptide glutamate (E)-isoleucine (I) (NEI), which is present in the same perikarya as MCH, and neuropeptide

glycine-glutamate, whose role as a functional peptide is unclear (25, 28).

Two receptors for MCH, MCH receptor 1 (MCH-R1) and MCH receptor 2 (MCH-R2), have been described to date (8, 17, 24, 33, 35). Both are seven-transmembrane domain G protein-coupled receptors that are expressed in many regions throughout the brain, including the hippocampal formation, olfactory regions, medial nucleus accumbens, ventromedial hypothalamic nucleus, and the lateral parabrachial nucleus (2, 16, 27, 36). Activation of MCH-R1 leads to inhibition of cAMP levels and phosphorylation of extracellular signal-regulated kinases 1 and 2 (ERK1/2) in various tissues (7, 15, 27). Interestingly, it has also been reported that MCH-R1 is expressed in the pituitary gland in both rodents and humans (34, 41) and that MCH neurons project to the median eminence region of the brain (5, 11), suggesting a role for MCH in mediating pituitary function.

The human pituitary normally produces six peptide hormones. These include growth hormone (GH), prolactin (PRL), thyroid-stimulating hormone (TSH), ACTH, luteinizing hormone (LH), and follicle-stimulating hormone, all of which are regulated by hypothalamic peptides. Several pituitary hormones have been linked to energy homeostasis by their capability to cause changes in body weight and composition. For example, GH deficiency in adults results in a marked increase in total body fat and a decrease in lean body mass, whereas GH excess leads to impaired glucose tolerance (12, 37). The thyroid axis has also been implicated in body weight regulation (21, 43), and hyperprolactinemia (14) as well as hypercortisolism (Cushing's syndrome) are associated with increased body weight and abnormal fat distribution.

The human fetal anterior pituitary gland gradually develops during the first and second trimesters, and by 25–26 wk of gestation, all hormone-producing cells are well differentiated (3). GH immunoreactivity is identified in human somatotrophs by 8 wk gestation (4), whereas distinct lactotrophs are only clearly identified after 24 wk. By 18–20 wk gestation, pituitary hormones respond, at least in part, to known physiological stimulators such as GH-releasing hormone (GHRH), somatostatin, dopamine, etc. (13, 30).

A few lines of evidence suggest that MCH, in addition to its role in appetite regulation, plays a role in several other physiological processes such as regulation of skin color in teleost fish (15) and interaction with the hypothalamic-pituitary axis in rodents, where it seems to affect thyroid hormone, ACTH, and

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LH secretion (9, 19, 20). However, there is no information on the effect of ppMCH derivatives on GH and PRL secretion, nor are there data regarding its effects on human pituitaries. The primary goal of this work was to study the effects of MCH on GH secretion from human pituitary tissue with the premise that MCH may exert some of its effects on energy balance, at least in part, via direct regulation of pituitary hormones. The ability of the ppMCH product NEI to modulate GH secretion was also assessed to further characterize potential biological roles for this peptide.

MATERIALS AND METHODS

Pituitary tissues. Fetal pituitary tissues were obtained from 21- to 25-wk-old human fetuses within 0.5–2 h of medical termination of the pregnancy. All studies were performed in accordance with the guidelines of the National Advisory Board on Ethics in Reproduction (10) after informed consent was obtained from the pregnant women. Normal adult pituitary tissue was harvested during post-mortem examination. Pituitary GH-secreting adenoma tissues were collected with informed consent during curative transphenoidal procedures. Pituitary tissues were either snap-frozen in liquid nitrogen (for RNA assays) or placed in culture medium for cell culture studies. Mouse pituitary tissues were obtained from 20-wk-old C57BL/6 males (Harlan), and experiments were carried out under the supervision and guidelines of the Sheba Medical Center animal welfare committee.

Expression of MCH-Rs in pituitary tissues. To assess the expression of MCH-Rs in human pituitary tissues, we performed RT followed by PCR amplification of MCH-R1 and MCH-R2 mRNAs in “normal” (healthy) and adenomatous human pituitary tissues. RNA was pretreated with DNase before the RT reaction to eliminate contaminating genomic DNA and then added to a 20- μ l RT reaction containing oligo(dT)₁₆ as a primer and SuperScript II (Life Technologies, Carlsbad, CA). RT reactions were initially incubated at 42°C for 50 min and then at 70°C for 15 min. The resulting cDNA and a negative water control were used for subsequent PCR amplification of MCH-R1 and MCH-R2 in the presence of 2 mM MgCl₂ and 5 U *Taq* DNA polymerase (Bioline, Randolph, MA). Amplifications were carried out for 40 cycles, with an initial denaturation step at 95°C for 5 min and a final 10-min extension step at 72°C. Each cycle consisted of denaturation at 94°C, annealing at 56°C, and elongation at 72°C, with each step lasting 30 s. The primers used were as follows: for MCH-R1, 5'-CCCGATAACCTCACTTCGG-3' and 5'-GACTATTGGCATCCATGGC-3'; for MCH-R2, 5'-GACGGTGTGAGAGTGTGTC-3' and 5'-GCAGCATCTGGCATCCTTAT-3'.

Pituitary cell cultures. All tissues were mechanically and enzymatically dispersed as previously described (32). Cells were re-suspended in DMEM-low glucose supplemented with 10% FBS and antibiotics. Approximately 1–5 \times 10⁴ cells/well were seeded in 48-well tissue culture plates (Costar, Cambridge, MA) in 0.5 ml of medium and incubated for 72–96 h in a humidified atmosphere of 95% air–5% CO₂ at 37°C. The medium was then changed to serum-free defined DMEM containing 0.2% BSA, 120 nM transferrin, 100 nM hydrocortisone, 0.6 nM triiodothyronine, 5 U/l insulin, 3 nM glucagon, 50 nM parathyroid hormone, 2 mM glutamine, 15 nM epidermal growth factor, and antibiotics. Cells were treated with either MCH, NEI (both obtained from Bachem), or GHRH (Sigma, St. Louis, MO) for 4 h, after which medium was collected and stored at –20°C for later hormone measurements. Wells treated with medium free of MCH, NEI, and GHRH served as controls. In the human studies, each experiment was done with fetal pituitary tissue originating from a single fetus or a single GH

cell adenoma and was repeated three times with different tissue donors, with five to six replicates used in each study. For rodent studies, each experiment represents pituitary tissues originating from 20-wk-old mice ($n = 10$), and these experiments were repeated two times, with five to six replicates used in each study.

Hormone assays. Human GH was measured by RIA (Diagnostic Products, Los Angeles, CA). Human PRL was measured using an immunoradiometric assay (IRMA) kit (Diagnostic Products). Human TSH was measured by IRMA (Diagnostic Products). Human ACTH was measured by Immulite chemiluminescence (Diagnostic Products). Because absolute hormone levels differed between fetal and adenoma specimens, all hormonal data were expressed as a percentage of control (100%). Mouse GH levels were measured by RIA (Linco Research, St. Charles, MO).

ERK1/2 phosphorylation assays. Pituitary GH adenoma cells were cultured in 3-cm plates and then serum starved (0.1% BSA) for 24 h. Cells were stimulated either with MCH (100 nM) alone or in combination with 25 μ M of the mitogen/extracellular signal-regulated kinase (MEK) inhibitor PD-98059 (Sigma) for the indicated times (0, 2, 5, and 10 min and 0, 2, and 5 min, respectively). After stimulation, cells were lysed in RIPA buffer (50 mM Tris·HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM sodium orthovanadate, 1 mM sodium fluoride, and 1 μ l/ml protease inhibitor cocktail) for 15 min at 4°C and centrifuged (15,000 g, 15 min, 4°C). The supernatants were collected, and equal amounts of protein (20 μ g) of each sample were separated by SDS-PAGE, transferred to nitrocellulose membranes, and blocked in 2% BSA. ERK1/2 phosphorylation was detected by probing blots with a monoclonal α -phospho-ERK1/2 antibody (DP-ERK; Sigma). Total ERK protein was detected using an α -ERK (ERK1 and ERK2) polyclonal antibody (Sigma), and signals were visualized with BCIP/NBT color development substrate (Promega, Madison, WI) or SuperSignal chemiluminescent substrate (Pierce, Rockford, IL).

Statistical analysis. Data are presented as means \pm SE from multiple experiments. Statistical analyses were performed using StatView (Abacus Concepts, Berkeley, CA). Differences between treatment groups were determined by one-way ANOVA and Fisher's test. A probability value of <0.05 was considered significant.

RESULTS

Expression of MCH-Rs in pituitary tissues. The expression of MCH-R1 was confirmed in different human pituitary tissues by RT-PCR. As shown in Fig. 1A, the expected 318-bp PCR product of MCH-R1 is expressed in both normal (fetal and adult) human pituitary tissues and GH cell-secreting adenoma tissue. To investigate whether the effects of MCH on pituitary GH secretion may be mediated by the MCH-R2, the expression of this receptor was also evaluated by RT-PCR. As shown in Fig. 1B, using human hypothalamus as a positive control, the expected 176-bp PCR product of MCH-R2 was not shown to be expressed in either normal or adenomatous human pituitary tissues.

Effects of MCH and NEI on GH release from dispersed human pituitary cell cultures. Human fetal pituitary cell cultures (derived from 21- to 25-wk-old fetuses) were incubated with either MCH (10 nM), NEI (10 nM), or a combination of the two in these concentrations for 4 h, after which the conditioned medium was collected and tested for GH content. GHRH (10 nM) was used as a positive control for GH stimulation, whereas serum-free medium was used as a negative control. As shown in Fig. 2A, MCH increased GH secretion from fetal pituitary cells by 62%, a magnitude comparable to the effect of the natural GH stimulator, GHRH, which in-

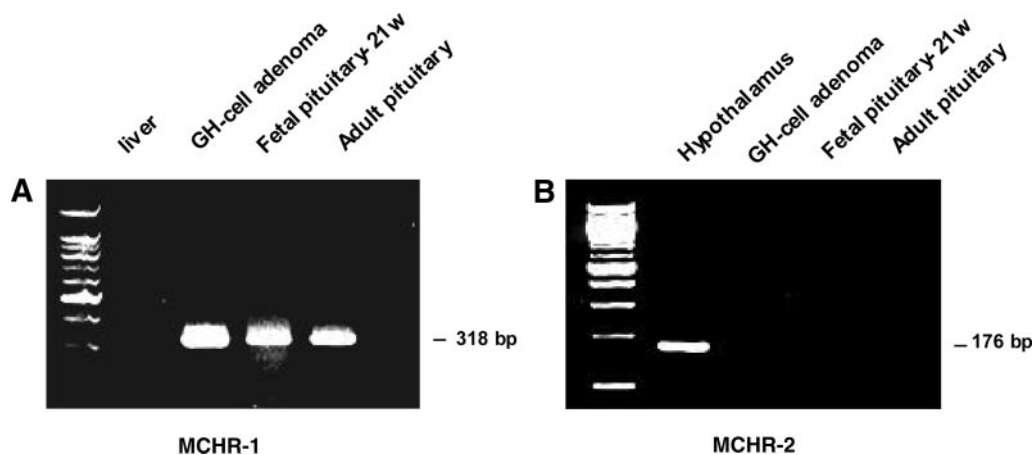


Fig. 1. Melanin-concentrating hormone receptor MCH-R expression in various human tissues as determined by RT-PCR. A: the predicted 318-bp PCR product of the MCH-R1 is depicted in fetal, adult, and growth hormone (GH)-secreting adenoma tissues. Human liver is included as a negative control. 21w, 21 wk. B: the predicted 176-bp PCR product of the MCH-R2 is absent in fetal, adult, and GH-secreting adenoma tissues. Human hypothalamus is included as a positive control.

creased GH secretion by 72% over basal levels. As illustrated in Fig. 2B, NEI increased GH secretion from cultured fetal pituitary cells by 124% vs. control, a magnitude comparable to the 155% increase seen with GHRH. Concomitant treatment with both MCH (10 nM) and NEI (10 nM) for 4 h had no additive or synergistic effects (data not shown). MCH (10 nM) had no effect on either TSH, ACTH, or PRL secretion in these cells (data not shown).

Effects of MCH and NEI on GH release from human GH-secreting pituitary adenomas. Cultured human GH adenoma cells were incubated with either MCH (10 nM) or NEI (10 nM) for 4 h. Serum-free medium was used as a negative control. Figure 3 shows the distinct responses of different cultured human GH-secreting adenomas to treatment with MCH and NEI. Treatment with both peptides induced GH secretion from these tumors; however, the extent of GH stimulation in response to treatment and the degree of sensitivity to one treatment vs. the other differed among the different tumors.

Effects of MCH on GH release from mouse pituitary cells. To further verify the ability of MCH to induce GH secretion from the pituitary gland, these experiments were repeated using primary cultures of mouse pituitaries. Mouse pituitary

cells were incubated with increasing doses of MCH (10, 100, or 1,000 nM) for 4 h. Serum-free medium was used as a negative control for GH stimulation. As shown in Fig. 4, MCH dose dependently increased mouse GH release by up to 40%.

Effect of MCH on ERK1/2 phosphorylation in human GH-secreting adenoma cultures. Previous studies have shown MCH-R1 signaling to be mediated in part via increased ERK1/2 phosphorylation (7, 15, 27). To determine whether GH stimulation in human pituitary tissues in response to MCH treatment reflects signaling via MCH-R1, we examined the ability of MCH to activate ERK1/2 in human GH-secreting adenoma cells. As shown in Fig. 5A, treatment of GH-secreting adenoma cells with 100 nM MCH induced a marked increase in the phosphorylation of ERK1/2 after 5 and 10 min of exposure. To confirm the specificity of the effects of MCH on ERK1/2 phosphorylation in GH-secreting adenoma cells, we tested whether this phosphorylation may be altered by adding the MEK inhibitor PD-98059. As shown in Fig. 5B, addition of the MEK inhibitor significantly decreased the ability of MCH to induce ERK phosphorylation in this tissue.

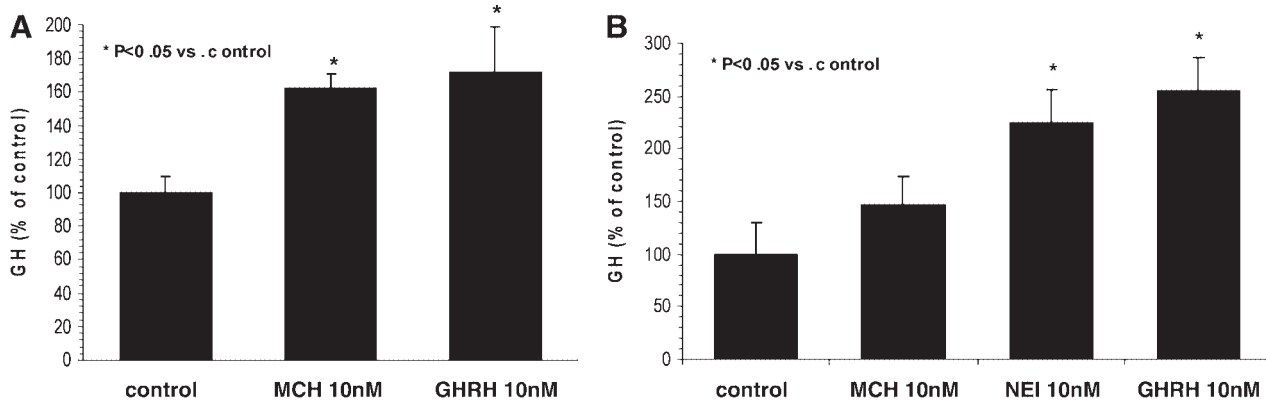


Fig. 2. GH secretion from human fetal pituitary tissues in response to treatment with MCH or neuropeptide glutamate (E)-isoleucine (I) (NEI). A: GH secretion from a 22-wk-gestation cultured fetal pituitary in response to treatment with 10 nM MCH or 10 nM GH-releasing hormone (GHRH) for 4 h. B: GH secretion from a separate 22-wk-gestation fetal pituitary cell culture in response to treatment with MCH, NEI, or GHRH for 4 h. Control cells were treated with vehicle solution. Each bar represents the mean + SE GH level of 6 wells and is representative of 3 independent experiments.

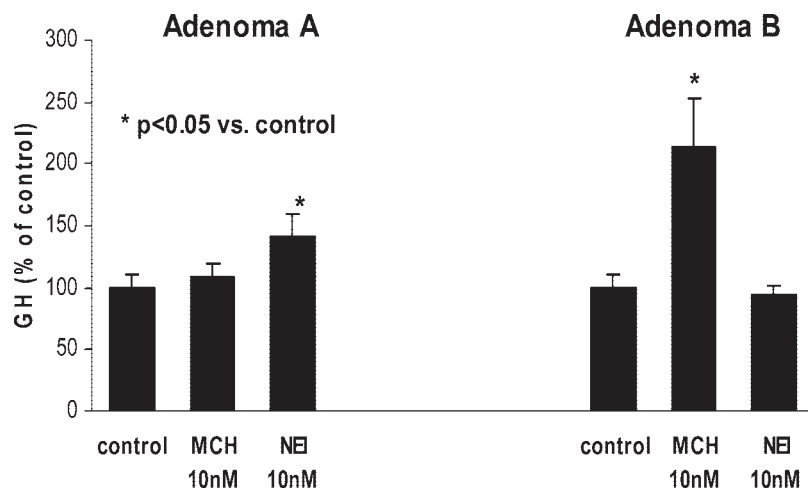


Fig. 3. GH secretion from two cultured human GH-secreting pituitary adenomas (adenoma A and adenoma B) in response to treatment with 10 nM MCH or 10 nM NEI compared with vehicle solution for 4 h.

DISCUSSION

MCH has established itself a role as an important mediator of energy homeostasis via regulation of food intake, energy expenditure, and locomotor activity. A few lines of evidence have suggested that MCH may influence the hypothalamic-pituitary axis. MCH-R1 was reported to be expressed in the pituitary gland of both rodents and humans (34, 41). In addition, projections from the lateral hypothalamus to the median eminence were described (5, 11), suggesting a role for MCH in the regulation of pituitary hormone secretion. Furthermore, MCH was recently detected around the hypophysial portal vessels, where it had a direct effect on gonadotropin release from the pituitary gland (9). Kennedy et al. have shown that both MCH and NEI decrease the secretion of TSH from rodent pituitary cells (20) and increase circulating levels of ACTH (19). Here, we confirm earlier reports showing expression of MCH-R1 in human and rodent pituitary glands (34, 41) and extend previous studies in this area by showing the lack of MCH-R2 expression in human pituitary tissues. We also report a novel effect of both MCH and NEI to induce GH secretion *in vitro* from human pituitary cell cultures and in murine animals. Although our studies clearly show that MCH and NEI stimulate GH secretion from human fetal pituitary cells, there seems to be a large degree of variability in the response of the tissues to GHRH stimulation, reflecting a large interexperimental

variation that in turn probably reflects variations in fetal age (21–25 wk gestation). This is supported by the differences in basal GH secretion from fetal pituitary tissues, ranging from 15.89 ± 1.42 to 30.65 ± 1.11 ng/dl in the different experiments. Another human pituitary tissue that responded with GH secretion to stimulation with MCH and NEI is GH cell adenoma. Again, like with the fetal tissues, there was a large variability in response to MCH/NEI treatment between the different tumors, with some reacting more to MCH and others being more sensitive to NEI. Although MCH-R expression was not tested in these tissues, we feel that tumors vary from each other in terms of their level of differentiation, which may lead to differences in the levels of receptor expression or the subreceptor signaling cascade, which in turn may lead to diverse hormone sensitivities. The effect of MCH on GH secretion was tested in mouse pituitary cell cultures, where a significant increase in GH secretion was observed, although the percent increase was much less than that seen in the human studies. This may reflect interspecies variation between rodents and humans in terms of the sensitivity of pituitary tissue to MCH or developmental differences such as fetal tissues being more sensitive to MCH compared with adult tissues. Further studies will be needed to resolve this issue.

Pituitary GH secretion is normally regulated by central and peripheral signals. The hypothalamic peptides GHRH and somatostatin stimulate and inhibit GH secretion, respectively. In the periphery, the stomach-derived hormone ghrelin stimulates GH release via the GH secretagogue receptor (40), whereas the liver-derived peptide IGF-I suppresses GH secretion. However, there is an increasing body of evidence suggesting that other factors may play an important role in GH regulation. Alba and Salvatori (1) have recently shown that GHRH gene-ablated mice had residual, albeit reduced, GH production in the pituitary (1), advocating a role for additional mediators of GH production. Moreover, in a very recent paper, Bjursell et al. (6) have shown that ghrelin administration to mice lacking the MCH receptor does not result in the typical increase in GH secretion, suggesting that the effects of ghrelin on GH secretion are mediated, at least in part, by MCH. Therefore, it seems that GH is regulated by multiple factors, including MCH and NEI, as indicated in the current study. However, the importance of MCH as a mediator of GH

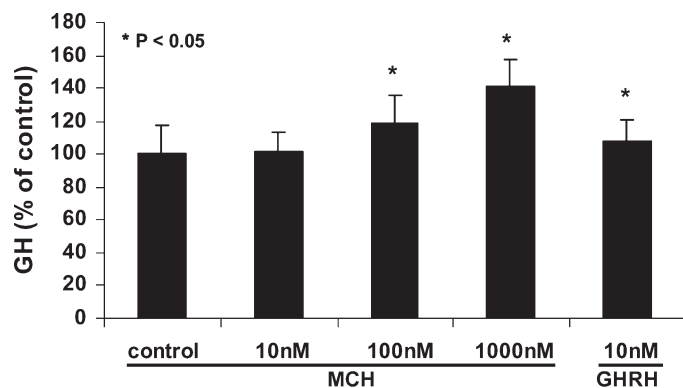


Fig. 4. GH secretion from mouse pituitary cell cultures in response to a 4-h incubation with increasing doses (10–1,000 nM) of MCH or GHRH (10 nM) compared with untreated cells.

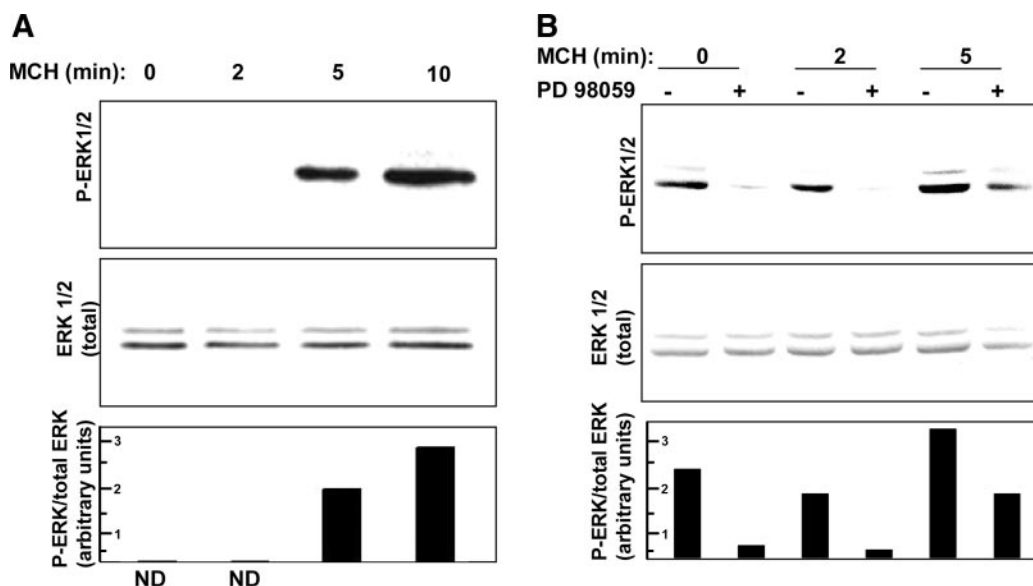


Fig. 5. MCH-induced phosphorylation (P) of extracellular signal-regulated kinases 1 and 2 (ERK1/2) and its inhibition by the mitogen/extracellular signal-regulated kinase inhibitor PD-98059 in human GH-secreting adenoma cells. *A*: cells were serum starved and incubated with or without 100 nM MCH for 2–10 min. *B*: cells were serum starved and incubated with 100 nM MCH, with or without PD-98059 for 2–5 min. Phosphorylation of ERK1/2 was detected with an α -phospho-ERK1/2 antibody. The total amount of ERK1/2 was detected with an α -ERK (ERK1 and ERK2) antibody. Results show changes from baseline in arbitrary units. ND, not detectable.

secretion under physiological conditions is yet to be determined and will merit further studies. It is worth noting that mice lacking either MCH or its receptor have normal birth weights and demonstrate no growth retardation throughout their lives, suggesting that growth and development are independent of MCH stimulation of GH secretion in these animals in which other GH secretagogues probably play a more critical role.

We also addressed potential signaling pathways mediating MCH-stimulated GH secretion. Because two MCH receptors have been described in humans, we first assessed the expression of both MCH-R1 and MCH-R2 in various human pituitary tissues and found that only MCH-R1 is expressed in these tissues. MCH-R1, like other G protein-coupled receptors, is capable of activating several intracellular kinases. MCH-R1 signaling has previously been shown to be mediated in part via increased phosphorylation of ERK1/2 in a number of cell lines (7, 15, 27). Consistent with these reports, we found that MCH-stimulated GH secretion was accompanied by a rapid increase in phosphorylation of ERK1/2, indicating that the stimulation of GH secretion indeed reflects MCH signaling in the pituitary. To our knowledge, this is the first report of MCH-induced phosphorylation of ERK1/2 in human cells endogenously expressing MCH-R1.

Our results add MCH to a growing list of appetite-regulating hypothalamic hormones affecting pituitary hormone secretion. For example, the appetite-stimulating hormone neuropeptide Y modulates the release of LH and inhibits PRL secretion from pituitary cells (18), and the appetite-suppressing hormone pro-opiomelanocortin, being the precursor of ACTH, may affect its production, suggesting that appetite-mediating hypothalamic peptides may regulate the hypothalamic-pituitary axis. In our studies, MCH did not seem to have an effect on the secretion of pituitary hormones other than GH from human fetal pituitary tissues. Although it is possible that the effect of MCH on

pituitary hormone secretion is restricted to GH, it is important to keep in mind that thyrotrophs and lactotrophs develop relatively late in human fetal life, and the inability of MCH to affect the secretion of either TSH or PRL may merely reflect the relative immaturity of the cells secreting these hormones. To our knowledge, no abnormalities in serum levels of pituitary hormone were reported in mice lacking either MCH or its receptor. Interestingly, in our study, NEI, a peptide that has some antagonistic effects on MCH, had similar effects to those of MCH on GH secretion. A similar trend was noted by Kennedy et al. (20), who showed that both of these peptides suppress TSH secretion from pituitary tissue. Further studies will be required to determine whether the functional antagonism between MCH and NEI is applicable to the pituitary gland.

In summary, we have shown that the human pituitary expresses a functional MCH receptor that, upon ligand binding, is capable of stimulating GH secretion. In addition, we propose a novel role for NEI, whose functions remain poorly defined, to stimulate GH secretion from pituitary cells. These data suggest that MCH may exert part of its effects on energy balance via direct pituitary hormone regulation. Thus this study provides insight into a potential interaction between two seemingly parallel systems, the appetite-regulating hypothalamic peptides and pituitary hormones, and also underscores the increasing complexity of the neuroendocrine systems involved in regulating energy balance.

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