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Endogenous receptor for melanin-concentrating hormone in human neuroblastoma Kelly cells

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Abstract

Melanin-concentrating hormone (MCH), a cyclic nonadecapeptide, is predominantly expressed in mammalian neurons located in the zona incerta and lateral hypothalamus. Current interest in MCH relates to its role in the control of feeding behaviour. Two receptors for MCH were recently found: MCH-R_1 and MCH-R_2. We show here by RT-PCR analysis and immunofluorescence studies that the human neuroblastoma cell line Kelly expresses MCH and MCH-R_1 but not MCH-R_2. In competition assays using ^{125}I-labelled MCH an inhibitory concentration 50% (IC_{50}) of 76 nM was determined for MCH, indicating a high affinity of Kelly cells for MCH. MCH induces mitogen-activated protein kinase (MAPK) phosphorylation in Kelly cells but no increase in the intracellular free Ca^{2+} concentration. This suggests that MCH signals via $G\alpha_i/G\alpha_0$ in these cells. The presence and functionality of MCH-R_1 renders this neuronal cell a very useful model for future structure–activity studies in a physiological environment mimicking the human brain for the evaluation of potential appetite-regulating drugs.

Keywords: Melanin-concentrating hormone; G protein-coupled receptor; Mitogen-activated protein kinase; Neuroblastoma

Melanin-concentrating hormone (MCH), initially isolated from teleost fish pituitary gland as a 17-amino acid peptide [1], is a potent mammalian orexigenic neuropeptide [2]. It is mainly produced by neurons of lateral hypothalamic area and zona incerta with projections widely distributed throughout the brain [3]. In 1999 and 2001, two receptor subtypes for MCH were discovered, namely MCH-R_1 [4–8] and -R_2 [9–14]. MCH-R_1 has been isolated from rodents and humans, whereas MCH-R_2 has thus far been identified only in humans [15]. Both are G protein-coupled receptors and are expressed in high concentrations in the brain [9,11,13]. Their expression in the periphery has not been generally agreed upon [12,13]. A complete review on MCH-R_1 and -R_2 expression sites is to be found in [16]. In cells transfected with MCH-R_1, MCH inhibits the production of forskolin-stimulated cyclic AMP (cAMP), activates mitogen-activated protein kinase (MAPK) [4–7], and induces an increase in intracellular free Ca^{2+} levels [17]. Pretreatment with pertussis toxin (PTX), which blocks $G\alpha_i/G\alpha_0$ proteins, abolishes the MCH-stimulated inhibition of cAMP production and MAPK activation, but only decreases the intracellular Ca^{2+} efflux [17]. Recently, human melanoma SK-MEL-37 cell line was shown to endogenously express functional MCH-R_1 [18]. In these cells, MCH inhibited forskolin-induced cAMP production and induced MAP kinase activation in a PTX-sensitive manner. However, in contrast to MCH-R_1-transfected cells, MCH did not increase intracellular free Ca^{2+} levels in SK-MEL-37 cells indicating, that it does not signal through G_q in this cellular environment. More recently, expression of MCH-R_1 was also reported for human melanocytes [19] and murine 3T3-L1 adipocytes [21]. In the latter, MCH significantly increased p44/42 MAPK and pp70 S6 kinase activities, providing evidence for a functional MCH signalling pathway in these cells [21].

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To better characterise expression and functionality of endogenously expressed MCH-R, we screened various human cell lines, namely HEK293 (embryonic kidney cells), HeLa (cervix adenocarcinoma cells), and Kelly (neuroblastoma cells) for the presence of MCH-R mRNA and protein. We report that Kelly cells express high levels of MCH-R_1 mRNA and protein and that MCH-R_1 is functional in these cells. The Kelly neuroblastoma cell line might serve as a useful model for studying the physiological MCH-R_1-mediated role of MCH in the brain.

Materials and methods

Cell culture. HEK293 and HeLa cells were obtained from Dr. Y. Saito and Dr. C. Kalberer, respectively. These cell lines were maintained in Dulbecco's modified Eagle's medium containing 4.5 g/L glucose, supplemented with 10% fetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 U/ml penicillin, and 50 μg/ml streptomycin (all from Gibco, Paisley, UK). Kelly cells, established in the Mount Sinai School of Medicine, New York [22], were obtained from Dr. D. Leppert and maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin (all from Gibco). Incubators were set at 37 °C with 5% CO_2 in a humidified atmosphere.

Semi-quantitative RT-PCR. Total RNA was extracted from cultured cells with a kit (RNeasy; Qiagen, Chatsworth, CA) and subjected to DNase I digestion (DNA-free kit; Ambion, Austin, TX) according to manufacturers' instructions. Total RNA from human adult normal brain was obtained from BioChain Institute (Hayward, CA). Total RNA (1 μg) was denatured at 65 °C for 5 min in the presence of 0.2 μg oligo $(dT)_{15}$; first-strand cDNA was produced at 37 °C for 60 min in the presence of 200 U M-MLV reverse transcriptase (Promega, Madison, WI), 1 mM dNTP, and 0.5 U ribonuclease inhibitor (Promega, Madison, WI) in a total volume of 20 μl, followed by a denaturation step of 5 min at 94 °C. Multiplex PCR was performed with cDNA corresponding to 25 ng total RNA, 200 μM dNTP, 0.05 μM of each β-actin primer, 1 μM of each of the other primers (Table 1), 1.5 mM $MgCl_2$, and 1 U HotStarTaq DNA polymerase (Qiagen, Chatsworth, CA) in a total volume of 20 μl. After a polymerase activation step at 94 °C for 15 min, 40 or 50 cycles at 94 °C (40 s), 62 °C (45 s), and 72 °C (45 s) were performed and followed by a final extension at 72 °C for 2 min. PCR products were resolved on a 1.5% agarose gel and visualised by ethidium bromide staining. Quantification of the intensity of PCR-amplified fragments was performed with a digital imaging system (Multi-Analyst Bio-Rad, USA) on more than three independent experiments and the results were standardised according to the corresponding β-actin value. The results are given as means ± SEM and statistical inference of the data was determined by an analysis of variance (ANOVA) with GraphPad Prism software. All PCR-amplified fragments were sequenced by Microsynth (Balgach, Switzerland) and corresponded to the targeted region.

Immunofluorescence studies. MCH-R_1 polyclonal antibody was generated in our laboratory. The antigenic peptide (H_2N-SNAQTADEERTESKG-COOH) corresponds to the carboxy-terminal end of the receptor and was produced by classical solid phase synthesis on a Pioneer automated peptide synthesiser. It was coupled to porcine thyroglobulin and injected into rabbits. IgG from both pre-immune and immunised blood sera was purified by passage through a protein A affinity column.

The cells, grown on 8-well glass slides (Lab-Tek Chamberslide, Nalge Nunc International, Naperville, IL), were washed twice with washing buffer (PBS containing 10% FCS and 10 mM NaN_3), fixed for 20 min in 50% acetone + 50% methanol at −20 °C, washed again, and incubated with 1:100 purified IgG (non-immune or immune) at room temperature for 30 min. Cells were then washed five times, incubated for 20 min with Alexa Fluor 488-conjugated goat anti-rabbit IgG (H + L) (1:200, Molecular Probes, Leiden, The Netherlands), washed twice, mounted with Fluorsave Reagent (Calbiochem, San Diego, CA), and examined under a Zeiss Axiophot Photomicroscope using a 40× objective.

Preparation of radioligand. Preparation of ^{125}I-labelled MCH has been described in detail elsewhere [23]. Briefly, 10 μg human MCH (Bachem, Bubendorf, Switzerland) was incubated for 50 min at room temperature with 1 mCi Na[^{125}I] (Amersham Pharmacia Biotech, Little Chalfont, UK) in 0.3 M Na-phosphate buffer, pH 7.4, and in the presence of 100 mU lactoperoxidase–biotin and 200 mU glucose oxidase–biotin (Sigma, St. Louis, MO). The reaction mixture was applied to an HBL extraction cartridge after removal of enzymes with streptavidin magnetic beads, and then the radioligand was eluted in methanol and stored at −70 °C. The radiopeptide was further purified by analytical HPLC on a C_{18} column (Symmetry 3.5 μm, 2.1 × 150 mm) using a gradient of acetonitrile from 10% to 100% for 39 min at a flow rate of 0.3 ml/min. The peak corresponding to monoiodinated peptide was collected and lyophilised overnight.

Receptor binding assay. Competition binding assays were performed as previously described [24]. Briefly, 10^6 cells were incubated at 20 °C for 2 h with 0.05 pmol ^{125}I-labelled MCH (~200,000 cpm) and competitor peptide in a 1:3 dilution series. Triplicate aliquots were layered onto silicone oil (1013 kg/cm^3) and cell-bound and unbound radioactivity was separated by centrifugation at 13,000 rpm and 4 °C for 2 min. Cell-bound radioactivity was measured in a γ-counter and data were analysed using GraphPad Prism software.

MAPK stimulation and Western blotting. Cells were grown in 6-well plates and serum-starved by culture in 0.5% FCS-containing medium for 24 h prior to stimulation at 37 °C with the selected compounds as indicated in the text and figure legends. Thereafter, cells were washed in PBS and lysed by the addition of 100 μl 1× SDS sample buffer

Table 1
Sequence of oligonucleotide primers used for RT-PCR, expected size (bp) of amplified products depending on the template

Targets	Forward primer (5′–3′)	Reverse primer (5′–3′)	cDNA size (bp)	Genomic DNA size (bp)
β-Actin	CAGCTCACCATGGATG ATGAT	CTCGGCCGTGGTGGTG AAGCT	623	1180
MCH-R1	CCCCGATAACCTCAC TTCG	GTGATGAGGGTGCA CATGG	300	1517
MCH-R2	CTGCCAGTGTGGTGGA TACAG	AACGTGTCAGTCGA AATGGTTG	347	>12,000
MCH	ATAAAGTTTCAAAG AACACAGGCT	ATACATCTGAGCAT GTCAAAATCT	226	No amplification[a]

[a] Forward primer is complementary to end of exon I and beginning of exon II; reverse primer is complementary to end of exon II and beginning of exon III of MCH gene.

(62.5 mM Tris–HCl, pH 6.8, 2% w/v SDS, 0.1% w/v bromophenol blue, 10% glycerol, 100 mM DTT, 5% v/v β-mercaptoethanol). Lysates were collected, denatured at 95 °C for 5 min, and electrophoresed on 12% reducing SDS–PAGE gels. Resolved proteins were electroblotted onto 0.45 μM nitrocellulose membrane (porablot NCL, Machery–Nagel, Düren, Germany). After blocking (1 h at room temperature) with 5% non-fat powdered milk in TBS-T (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, and 0.1% v/v Tween 20) membranes were incubated overnight at 4 °C with polyclonal anti-phospho-p44/42 MAPK (Cell Signaling Technology, Beverly, MA) at a 1:1000 dilution in TBS-T + 5% BSA. After three 5-min washes in TBS-T, membranes were incubated for 1 h at room temperature with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Cell Signaling Technology, Beverly, MA) at 1:2000 in TBS-T + 5% milk. Immunoreactive proteins were detected by chemoluminescence (SuperSignal West Pico kit, Pierce, Rockford, IL) and exposure to Kodak Biomax films (Eastman Kodak Company, Rochester, NY).

Results

Semi-quantitative RT-PCR

RT-PCR analysis showed that MCH and MCH-R_1 mRNA, but not MCH-R_2 mRNA, were expressed in Kelly cells (Figs. 1A and B). Compared with HEK293 and HeLa cell lines, Kelly cells expressed MCH-R_1 mRNA 10 times more abundantly and at a level comparable to that found in the human brain as positive control (Fig. 1D). Both MCH-R_1 and -R_2 mRNA were detectable from 35 PCR cycles onwards in HEK293 and HeLa cell lines (Fig. 1A; 40 cycles). However, we were unable to amplify MCH-R_2 mRNA from Kelly cells, even with extended PCR (50 cycles, Fig. 1C; 60 cycles, data not shown). The transcript for the hormone MCH was found in Kelly cells and at a level comparable to that in the human brain, whereas even with extended PCR neither HEK293 nor HeLa yielded a positive signal (50 cycles, Fig. 1B).

MCH-R_1 immunolabelling

Positive immunofluorescence staining for MCH-R_1 was observed in Kelly cells (Fig. 2A). Staining was restricted to the cell surface, mainly on the cellular processes. Specificity of our polyclonal antibody for MCH-R_1 was confirmed by the negative control (using IgG from pre-immune serum) where a faint global staining was evident (Fig. 2B). In HEK293 cells the faint global immunofluorescent signal with anti-MCH-R_1 (Fig. 2C) was similar to that obtained with IgG from pre-immune serum (Fig. 2D).

Receptor binding assay

^{125}I-labelled MCH competition binding experiments (Fig. 3 and Table 2) revealed that Kelly cells have a high affinity for the radioligand (IC_{50} of ~76). The affinity of HEK293 cells for ^{125}I-labelled MCH was 3-fold less

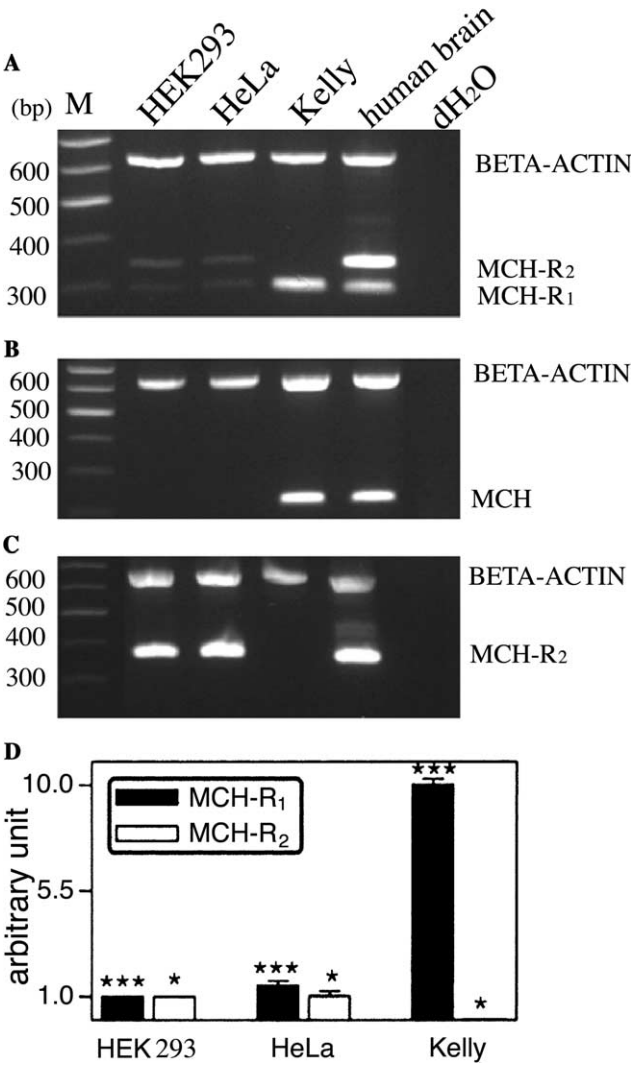

Fig. 1. Expression of MCH, MCH-R_1, and MCH-R_2 in HEK293, HeLa, and Kelly cells. (A–C) PCR amplification of fragments corresponding to MCH-R_1, MCH-R_2, MCH, and β-actin cDNAs; no genomic amplification occurred. Human brain cDNA or no cDNA (dH_2O) was used as positive and negative controls, respectively. (D) Quantification of the intensity of PCR bands, standardised with β-actin. Data are means ± SEM values from six separate experiments. $^{***}p < 0.001$; $^{*}p < 0.01$.

(IC_{50} of ~240). In HEK293 cells overexpressing the rat MCH-R_1 we have determined an IC_{50} of 48.82 ± 9.5 nM (data not shown), consistent with a high affinity of MCH-R_1 for the radioligand.

Signalling assays

We first examined the intracellular Ca^{2+} response to MCH using fluorimetric imaging plate reader (FLIPR) assay. MCH, even at a high concentration of 1 μM, did not elicit an increase in the intracellular free Ca^{2+} concentration in either HEK293 or Kelly cells. Functionality of the Ca^{2+} system in these cell lines was confirmed by the positive control (stimulation with 40 mM $CaCl_2$)

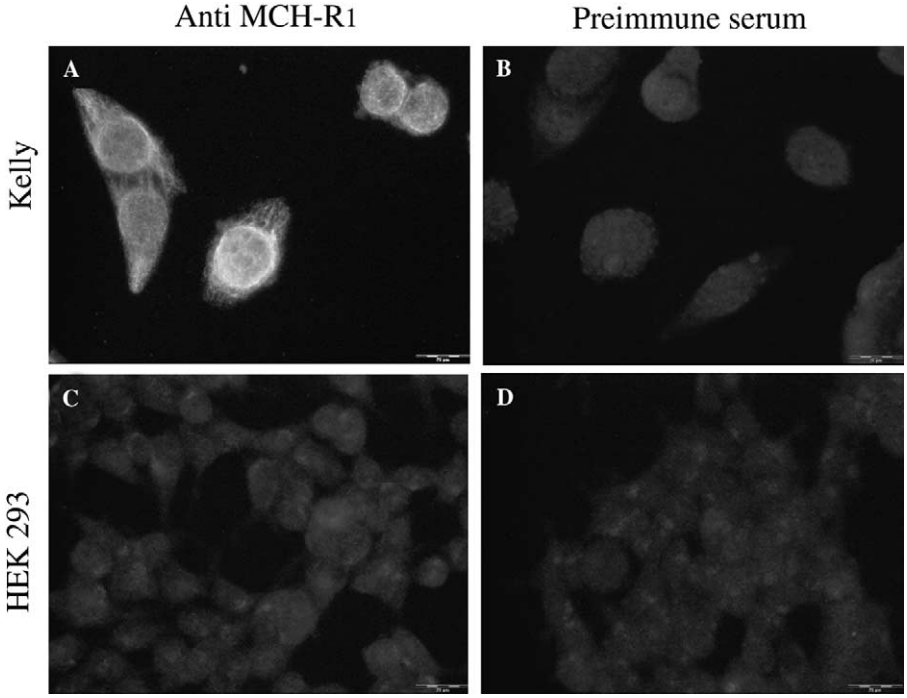

Fig. 2. MCH-R_1 immunostaining on Kelly (A) and HEK293 cells (C) IgG from preimmune serum served as the negative immunostaining control (B and D). Calibration bar represents 20 μm.

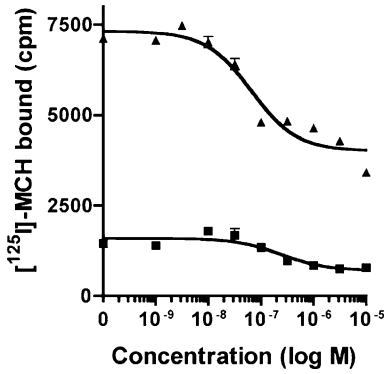

Fig. 3. ^{125}I-labelled MCH competition binding curves performed with Kelly (▲) and HEK293 (■) cells. Unlabelled MCH was used as the competitor.

whereby a strong response was produced (data not shown).

The ability of MCH to activate MAPK signalling was examined by Western blot assay. In Kelly cells MCH stimulated the phosphorylation of p42 (= ERK2) and p44 (= ERK1) forms of MAPK in a time-dependent manner (Fig. 4A, lanes 0–5–10). Phosphorylation of p42/p44 returned to basal values within 30 min (data not shown). Maximum phosphorylation of MAPKs was obtained after a 10-min stimulation with MCH at 333 nM. A 24-h preincubation of Kelly cells with pertussis toxin (PTX; 400 ng/ml) inhibited MCH-stimulated MAPK phosphorylation (Fig. 4A, lane PTX). MCH was unable to induce MAPK phosphorylation in HEK293 cells, although MAPK signalling in these cells is functional as demonstrated by the response to the positive control with 10% FCS (Fig. 4B).

Table 2
Binding affinities of MCH on HEK293 and Kelly cells

Cell line	IC_{50} (nM)	Specific binding (%)
HEK293	240.5	46
Kelly	76.08 ± 9.9	57.6 ± 5.6

Inhibitory concentration 50 (IC_{50}) and percentage of specific binding are expressed as mean ± SEM in the case of $n = 2$.

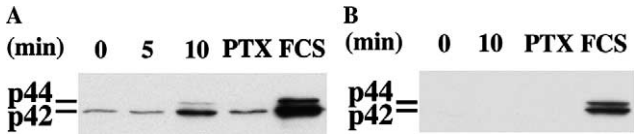

Fig. 4. Immunoblot analysis of phosphorylated MAPK, p42/44. (A) Kelly and (B) HEK293 cells were stimulated for the indicated times (min) with 333 nM MCH (lanes 0–5–10) and with 10% FCS for 10 min (lane FCS). Kelly cells were preincubated with pertussis toxin (PTX at 400 ng/ml for 24 h and cells were subsequently stimulated with 333 nM MCH for 10 min (lane PTX). Pictures are representative of three independent experiments.

Discussion

Thus far endogenous expression of MCH-R_1 has only been reported for major cell lines and tissues of peripheral origin, i.e., the human melanoma SK-MEL-37

cell line [18], human skin and primary melanocytes [19], human immune cells [20], and murine 3T3-L1 adipocytes [21]. Nevertheless, MCH as well as its receptor subtypes 1 and 2 are predominantly expressed in the central nervous system [3,16]. Since it is rather difficult to obtain and maintain pure cultures of neurons from human brain, well-characterised human neuroblastoma cell lines have served as useful alternative experimental models for human neurons. For example, the transcriptional effect of the complex consisting of α1-antichymotrypsin and Alzheimer's peptide Abeta (1–42) was studied in the Kelly neuroblastoma cells [25]. Human neuroblastoma cell lines such as SH-SY5Y and Kelly both represent interesting models for analysis of complement biosynthesis by human neurons [26]. We therefore decided to screen the Kelly neuroblastoma cell line together with two non-neuronal control cell lines, HEK293 and HeLa, for the presence of MCH-R mRNA and protein by RT-PCR, immunofluorescence, competition binding, and functional assays.

We demonstrate that Kelly cells express mRNA for MCH at a similar level as the human brain. Detection of MCH mRNA in HEK293 or HeLa cell lines was only possible with extended RT-PCR. It is perhaps not surprising that expression of MCH-R_1 in Kelly cells is at least 10-fold greater than that in the non-neuronal HEK293 and HeLa cell lines, since the major site of MCH expression is the central nervous system. Interestingly, we found that Kelly cells expressed only MCH-R_1 and since we were unable to detect any signal for MCH-R_2 mRNA even after up to 60 PCR cycles, this cell line is apparently devoid of its expression. It has been reported that MCH-R_1 is more abundant and has a wider distribution pattern than MCH-R_2 [9,11,13]; reviewed in [16].

We could determine only low levels (from 35 PCR cycles onwards) of MCH-R_1 and MCH-R_2 transcripts in HEK293 and HeLa cells. One study has reported HeLa cells to be negative for MCH-R_1 mRNA expression by RT-PCR [27]. The reason for this discrepancy is that in the previous study [27] the number of PCR cycles did not exceed 33 cycles (K. Takahashi, personal communication). There is also a similar discrepant report of an absence of MCH-R_1 mRNA in HEK293 cells [28], explainable perhaps by the use of Northern blotting for detection, a method that is less sensitive than the RT-PCR applied in this study. In HEK293 cells we could neither detect MCH-R_1 protein by immunostaining nor demonstrate MAPK activation. On the other hand, in spite of the low level of MCH-R_1 mRNA detected in HEK293 cells and correspondingly low translation into protein, we were able to prove specific binding of ^{125}I-labelled MCH radioligand. These latter data demonstrate the good affinity of ^{125}I-labelled MCH radioligand for its receptor as well as the sensitivity of the radioimmuno-assays.

The present study confirms findings of a previous study in SK-MEL-37 melanoma cells [18] that showed that endogenous human MCH-R_1 does not signal through $G\alpha_q$ in the native cellular environment. In SK-MEL-37 melanoma [18] and Kelly neuroblastoma cells [this study], MCH was unable to elicit an increase in intracellular free Ca^{2+} concentrations but rather inhibited forskolin-stimulated cyclic AMP accumulation [18] and induced MAPK phosphorylation [18, this study]. Furthermore, as in SK-MEL-37 cells [18], MCH-induced MAPK kinase activation in Kelly cells was sensitive to inhibition by PTX, thereby confirming that endogenous MCH-R_1 is coupled to $G\alpha_i/G\alpha_0$ proteins.

In conclusion, we have demonstrated the presence of functional MCH-R_1 in the human Kelly neuroblastoma cell line. In this endogenous environment and as opposed to CHO [5,17] or HEK293 [4,6,29] overexpression models, MCH-R_1 does not respond to MCH stimulation with an increase of intracellular Ca^{2+} concentration. In the Kelly cells, MCH-R_1 is coupled to PTX-sensitive $G\alpha_i/G\alpha_0$ proteins, leading to phosphorylation of MAPK, p42/44. Furthermore, endogenous Kelly MCH-R_1 binds ^{125}I-labelled MCH satisfactorily. The Kelly cell line might represent a valuable experimental model for future structure–activity studies in a physiological environment mimicking the human brain for the evaluation of potential appetite-regulating drugs.

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