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CLINICAL STUDY

Melanocortin 4 receptor distribution in the human hypothalamus

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Abstract

Objective: The melanocortin 4 receptor (MC4R) is an essential regulator of energy homeostasis and metabolism, and *MC4R* mutations represent the most prevalent monogenetic cause of obesity in humans known to date. Hypothalamic MC4Rs in rodents are well characterized in neuroanatomical and functional terms, but their expression pattern in the human hypothalamus is unknown.
Design and methods: To determine the topographic distribution and identity of cells expressing *MC4R* mRNA in the human hypothalamus, locked nucleic acid *in situ* hybridization was performed on nine human postmortem hypothalami. In addition, co-expression of *MC4R* with glial fibrillary acidic protein (GFAP), vasopressin/oxytocin (AVP/OXT), corticotropin-releasing hormone (CRH), neuropeptide Y (NPY), agouti-related protein (AgRP), and α-melanocyte stimulating hormone (α-MSH) was examined.
Results: Most intense *MC4R* mRNA expression was present in the paraventricular nucleus (PVN), the supraoptic nucleus (SON), and the nucleus basalis of Meynert. Most *MC4R*-positive cells in the SON also expressed AVP/OXT. Co-expression with AVP/OXT in the PVN was less abundant. We did not observe co-expression of *MC4R* mRNA and GFAP, CRH, NPY, AgRP, or α-MSH. However, fiber-like staining of NPY, AgRP, and α-MSH was found adjacent to *MC4R*-positive cells in the PVN.
Conclusion: Expression of *MC4R* mRNA in the human hypothalamus is widespread and in close approximation to endogenous MC4R binding partners AgRP and α-MSH.

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Introduction

The melanocortin 4 receptor (MC4R) plays an essential role in the maintenance of energy balance and is stimulated by endogenous melanocortins. MC4R has high affinity for α-melanocyte stimulating hormone (α-MSH) (1), while the receptor is inhibited by endogenous agouti-related protein (AgRP) (2). Stimulation of MC4R decreases food intake and increases energy expenditure (3, 4). Less is known about the regulation of hypothalamic *MC4R* expression.

The important role that MC4R plays in energy homeostasis is underlined by several observations: in rodents as well as in humans, heterozygous and homozygous mutations in *MC4R* lead to severe obesity. *MC4R* heterozygosity accounts for 2.5–6% of all cases of morbid childhood-onset obesity (5, 6, 7) and more than 90 obesity-associated *MC4R* mutations have been identified to date (8). In addition to obesity, mutations in one or both alleles of *MC4R* in humans are also associated with elevated fasting insulin levels, elevated blood glucose levels, enhanced linear growth, and incompletely suppressed GH secretion, as well as a reduction in blood pressure and heart rate, while a decreased prevalence of hypertension is also associated with mutations in *MC4R* (9, 10, 11, 12). To obtain more insight into the role of MC4R in hypothalamic melanocortin signaling, a better understanding of the underlying functional neuroanatomy is crucial.

In spite of the severe metabolic phenotype and the high prevalence of MC4R abnormalities, no distribution studies that map *MC4R* expression in the human brain have been published. mRNA analysis in mice and rats (13, 14), as well as transgenic approaches (15), has shown that *MC4R* expression is restricted to the CNS and many functional experiments underline the importance of MC4R function in the hypothalamus (16, 17). Therefore, we set out to examine for the first time the expression of the MC4R in the human hypothalamus. We chose a locked nucleic acid (LNA) *in situ* hybridization approach, as reliable MC4R antisera for immunohistochemistry in human brain are not available.

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Materials and methods

Subjects

Postmortem hypothalamic tissue specimens of nine individuals were studied; both men and women were included. Hypothalami were selected from subjects without any known neurological, neurodegenerative, or psychiatric disease. Clinicopathological data are described in Table 1. All brain material was obtained from The Netherlands Brain Bank at The Netherlands Institute for Neuroscience (director Dr I Huitinga) in accordance with the formal permissions for brain autopsy and for the use of human brain material and clinical information for research purposes.

LNA probe

We designed an LNA-2′-*O*-methyl-RNA probe specific for the human *MC4R* mRNA sequence. We used LNA-2′-*O*-methyl-RNA nucleic acid analogues because they are stable and have high hybridization affinity and have been applied in human brain successfully (18, 19). We used an antisense probe 5′-lTmUmGlCmUmGlTmGmClAmG-mUlCmUmGlTmAmAmC-3′ complementary to bases 478–496 of the human melanocortin 4 receptor (GenBank ref NM005912.2) and the corresponding sense probe: 5′-lGmUmUlAmCmAlGmAmClTmGm-ClAmCmAlGmCmAlA-3′, wherein 'm'=2′-*O*-methyl-RNA (mA, mG, mC, and mU) and 'l'=LNA bases. Probes were FAM tagged at the 5′ end and custom ordered (Ribotask, Langeskov, Denmark). Specificity of the probe was supported using the sense probe and by testing of a concentration gradient ranging from 500 pM to 100 nM.

Distribution study in situ hybridization and immunohistochemistry

Hypothalami were dissected at autopsy and fixed in 10% (v/v) phosphate-buffered formalin at room temperature (RT) for 24–87 days (see Table 1). After dehydration in graded ethanol series, tissues were cleared in toluene and embedded in paraffin. Coronal serial sections (6 μm) were cut over the entire rostro-caudal axis. We collected every 100th section, spanning the entire hypothalamus, and mounted them on Superfrost plus slides (Menzel Glaser, Braunschweig, Germany) and subsequently dried them for at least 2 days at 37 °C. Sections were deparaffinized and rehydrated by xylene and a series of gradient alcohols followed by rinsing in PBS. Sections were pretreated for 10 min in the microwave at 700 W in PBS, which was followed by a 90-min prehybridization in a humidified chamber at 55 °C in a hybridization mixture (hymix) with final concentrations of 50% (v/v) deionized formamide, 600 mM NaCl, 10 mM HEPES, 5× Denhardt's, 1 mM EDTA, and 40 μg/ml fish sperm (Invitrogen). Probe was diluted in hybridization buffer to a final concentration of 50 nM, denatured at 95 °C for 5 min, and cooled on ice. Sections were hybridized in this hybridization mix overnight at 55 °C and subsequently washed for 5 min each in 2× SSC, 0.5× SSC, and 0.2× SSC at 55 °C and for 5 min in TBS at RT. Next, sections were incubated in anti-FAM-Alkaline Phosphatase (Roche) 1:4000 in SUMI (0.25% (w/v) gelatin and 0.5% (v/v) Triton X-100 in TBS, pH 7.6, for 3 h at RT. Slides were washed 5 min in buffer 1 (100 mM Tris, 150 mM NaCl at pH 7.5) twice, and after a prewash in 100 mM Tris–HCl, pH 9.5, 100 mM

Table 1 Clinicopathological data.

NBB number	Age (years)	Sex	PMD (h)	Fix (days)	BMI	Clinical diagnosis	Cause of death	Co-staining
1980-008	35	F	8:00	26	ND	Acute lymphoblastic leukemia	Multi-organ failure	α-MSH, AVP/OXT
1998-237	38	M	10:45	37	ND	Wegener's disease, renal failure	Multi-organ failure	
1998-161	60	F	8:06	87	22.9	Metastasized ovarian cancer	Respiratory failure	α-MSH AgRP AVP/OXT CRH GFAP
1998-127	56	M	5:25	35	29.5	Cardiovascular disease, type 2 diabetes, sepsis	Ventricular fibrillation	α-MSH AgRP
1994-076	78	M	8:25	24	ND	Bechterew's disease	Heart failure	
2001-005	77	F	19:45	36	32	Non-Hodgkin lymphoma	Heart failure/ multi-organ failure	α-MSH AgRP AVP/OXT CRH NPY
2000-007	85	M	15:10	35	ND	Recurrent myocardial infarction, asthma cardiale	Heart failure	α-MSH AgRP CRH NPY
Co-staining study only								
2000-090	70	M	7:45	34	ND	Metastasized bladder carcinoma	Respiratory insufficiency	α-MSH, AgRP, AVP/OXT, GFAP
2001-021	82	M	7:40	32	27.8	Ischemic heart disease	Heart failure	α-MSH, AgRP, CRH, GFAP, NPY

F, female; fix, fixation time; M, male; ND, not determined; PMD, postmortem delay.

NaCl, and 5 mM $MgCl_2$, the color was developed using NBT-BCIP solution (337.5 μg/ml NitroBlue Tetrazolium Chloride (Sigma), 175.4 μg/ml 5-bromo-4-chloro-3-indolyl phosphate (Roche), toluidine salt, 240 μg/ml levamisole in 100 mM Tris–HCl, pH 9.5, 100 mM NaCl, and 5 mM $MgCl_2$) for 3 h under dark conditions. Sections were then washed in distilled water, incubated in methanol (Sigma) for 5 min, and rinsed again in distilled water. Finally, the sections were coverslipped with Aquamount (Merck) and stored at 4 °C.

Co-expression MC4R *mRNA and immunohistochemistry*

To further identify cell types expressing the MC4R, sections were hybridized and subsequently washed twice in TBS. Sections were incubated in primary antibodies diluted in SUMI (1:100 000 rat monoclonal anti-corticotropin-releasing hormone (anti-CRH) antibody, 'PFU-83' (20), or 1:500 vasopressin/oxytocin (AVP/OXT) (Truus (21, 22)) for 1 h at RT followed by an overnight incubation at 4 °C. Alternatively, a blocking step for 1 h at RT in 5% (w/v) milk in TBS preceded a primary antibody incubation in 5% (w/v) milk-SUMI for 1 h at RT followed by overnight incubation at 4 °C (1:1000 neuropeptide Y (NPY) (Niepke 091188 (23)), 1:1500 AgRP (Phoenix Pharmaceuticals, Burlingame, CA, USA), 1:20 000 α-MSH (Chemicon, Temecula, CA, USA), and 1:1000 glial fibrillary acidic protein (GFAP; Dako, Glostrup, Denmark)). Sections were washed in TBS and incubated in the appropriate biotinylated secondary antibody 1:400 in SUMI for 1 h. After washing in TBS, sections were incubated for 1 h in avidin–biotin complex (1:800 in SUMI; Vector Laboratories, Burlingame, CA, USA) and subsequently rinsed in 0.2 M glacial acetic acid. Finally, sections were incubated in glacial acetic acid buffer (0.2 M acetic acid and 0.2 M sodium acetate, pH 5.2) with 0.015% (w/v) hydrogen peroxide and 0.5 mg 3-amino-9-ethylcarbazole (AEC)/ml. Reaction times were determined experimentally. After stopping the reaction in water, sections were coverslipped using Aquamount (Merck) and stored at 4 °C.

Characteristics of primary antibodies

All antibodies used and their immunogens are reported in Table 2. Magnocellular neurosecretary neurons of the paraventricular nucleus (PVN) and supraoptic nucleus (SON) were stained with an AVP antibody (Truus), which was raised in rabbit against AVP in its processed form, although this antibody has been reported to cross-react with OXT (20, 21). CRH staining specificity was supported by absence of staining using the pre-immune serum (22). In addition, staining disappeared after pre-adsorption with CRH and was unaffected by cross-adsorption using AVP (22). AgRP staining disappeared after pre-adsorption with AgRP and was not affected by cross-adsorption using the NPY peptide (23). The α-MSH antibody was raised against the α-MSH C-terminal, which is modified in α-MSH-free acid, and absent in ACTH, minimizing cross-reaction with other POMC products. Staining was abolished after pre-adsorption with the α-MSH peptide (24). NPY antibody specificity was previously tested by pre-immune staining and antibody pre-adsorption (23). GFAP showed exclusive glial staining. Finally, we tested omission of all primary antibodies, which did not reveal any staining.

Analysis

Distribution study Staining intensity was scored by visual inspection in a semiquantitative fashion as follows: − (no staining), +/− (few positive cells showing light staining intensity), + (the majority of cells are positive and show a moderate to strong signal), ++ (the majority of cells are positive and staining intensity is high). We did not use a nuclear marker;

Table 2 Antibody characteristics.

Antibody	Immunogen	Host	Manufacturer, Catalog #
GFAP	GFAP isolated from bovine spine	Rabbit polyclonal	DakoCytomation, Denmark, Z 0334
CRH	CRH-bovine thyroglobulin conjugate	Rat monoclonal	Prof. F J H Tilders (Free University of Amsterdam) (Amsterdam): PFU 83
AVP/OXT	Synthetic peptide Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH_2 (V-9879, Sigma) coupled to thyroglobulin (Sigma) with glutaraldehyde (Merck)	Rabbit polyclonal	Netherlands Institute for Neuroscience (Amsterdam): #Truus, C.P.180985
NPY	Porcine NPY (1–36) (Sigma) coupled to thyroglobulin (Sigma) with glutaraldehyde (Merck)	Rabbit polyclonal	Netherlands Institute for Neuroscience (Amsterdam): #Niepke C.P.261188
α-MSH	α-MSH coupled to bovine thyroglobulin	Sheep polyclonal	Millipore, MAb Technologies, Inc., Stone Mountain, GA, USA: Mab5087
AgRP	Human AGRP (83–132)	Rabbit polyclonal	Phoenix Pharmaceuticals, Inc., Belmont, CA, USA: #H-003-57

GFAP, glial fibrillary acidic protein; CRH, corticotropin-releasing hormone; AVP/OXT, vasopressin/oxytocin; NPY, neuropeptide-Y; α-MSH, α-melanocyte stimulating hormone; AgRP, agouti-related protein.

however, nucleoli of negative cells were visible under the light microscope. Every 100th 6 μm section was scored, such that every nucleus was studied in a systematic manner covering all levels over the rostro-caudal axis. Two independent researchers assigned scores visually. Scores were compared and found to be highly similar between researchers. If scores differed, the researchers reevaluated the sections together until consensus was reached.

Co-localization study Co-localization of *MC4R* and other peptides was examined in three individuals per peptide (Table 1), with three sections per individual. For co-localization studies, we sampled the central part of the nucleus. To support visual scoring of co-localization in an objective manner, we applied spectral analysis. Spectral imaging identifies colors based on their spectral characteristics, enabling extraction and visualization of the separate colored reaction products (25). Spectral analysis was performed using a CRi Nuance FX camera and software, Image pro (Media Cybernetics, Silver Spring, MD, USA), in combination with software developed at the Netherlands Institute for Neuroscience. A spectral library of the individual spectra of NBT-BCIP and AEC was made by collecting light in 'cubes' of 20 nm along a 200–600 nm spectrum, thus obtaining distinct peaks in the resulting individual light absorption graphs for the two substrates. The spectral library was then applied to image cubes (from 200 to 600 nm) of the double-stained slides, which were then spectrally unmixed into individual black and white images, representing the localization of each of the reaction products. These were reverted to pseudo-colored images using the Nuance software to enhance color separation and visualization of co-localization.

Results

Specificity

We designed an LNA probe to visualize *MC4R* specifically and observed the same distribution pattern using a dilution series supporting specificity (Fig. 1). Staining specificity was further supported by the absence of staining with the complementary sense probe together with the antisense probe (see insets in Fig. 2).

Distribution of MC4R *mRNA in the human hypothalamus*

To obtain the expression pattern of *MC4R* mRNA, three women and four men were studied (characteristics in Table 1). We observed a strong interindividual variation in *MC4R* mRNA staining intensity, which showed no obvious correlation with sex or age, although these studies were not designed to investigate such effects.

Expression of *MC4R* mRNA within the hypothalamus was found to be widespread (Figs 2 and 3). Contrary to the strong interindividual variation in staining intensity, staining distribution was highly similar between subjects (Table 3). Staining was most intense in the magnocellular neurons of the SON, the PVN, and in the nucleus basalis of Meynert (NBM). *MC4R* was also expressed – although to a lesser extent – in the medial preoptic area (MPO), the lateral septal nucleus (LS), the tuberomammillary nucleus (TMN), the bed nucleus of the stria terminalis (BST), and the mammillary nucleus (MM). Least intense staining and fewer *MC4R*-positive cells were found in the ventromedial hypothalamic nucleus (VMN), the dorsomedial hypothalamic nucleus (DMN), the lateral hypothalamus (LH), and the nucleus tuberalis lateralis (NTL). We found no *MC4R* expression in the suprachiasmatic nucleus (SCN), infundibular nucleus (IFN; the human equivalent of the arcuate nucleus), or in the medial septal nucleus (MS). The sexually dimorphic nucleus (SDN) and diagonal band of Broca (DBB) were clearly labeled in one patient (NBB number 1998-161), but not in others, for which we found no clear explanation.

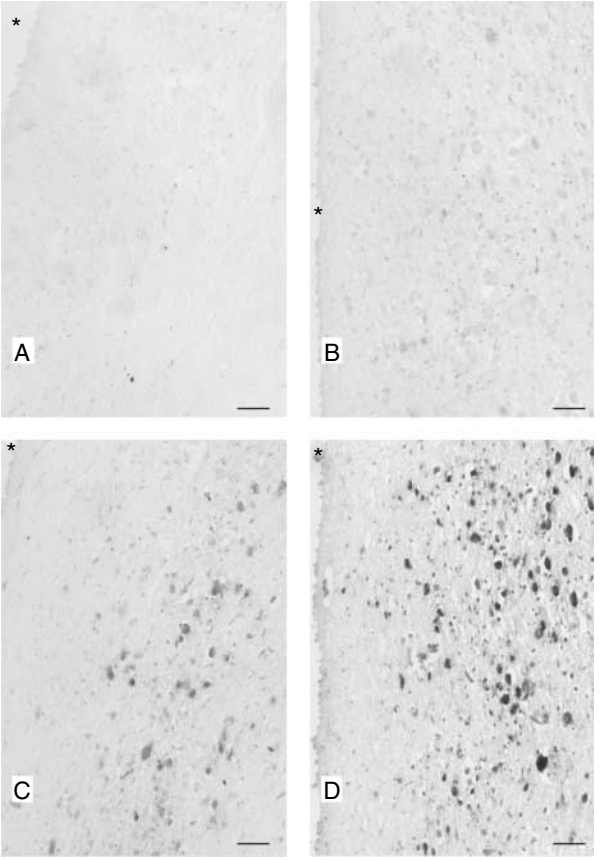

Figure 1 *MC4R* probe concentration range. (A) 0.5 nM antisense probe in PVN of patient 2000-007. (B) 10 nM antisense probe in PVN of patient 2000-007. (C) 50 nM antisense probe in PVN of patient 2000-007. (D) 100 nM antisense probe in PVN of patient 2000-007. Scale bar represents 50 μM; *third ventricle.

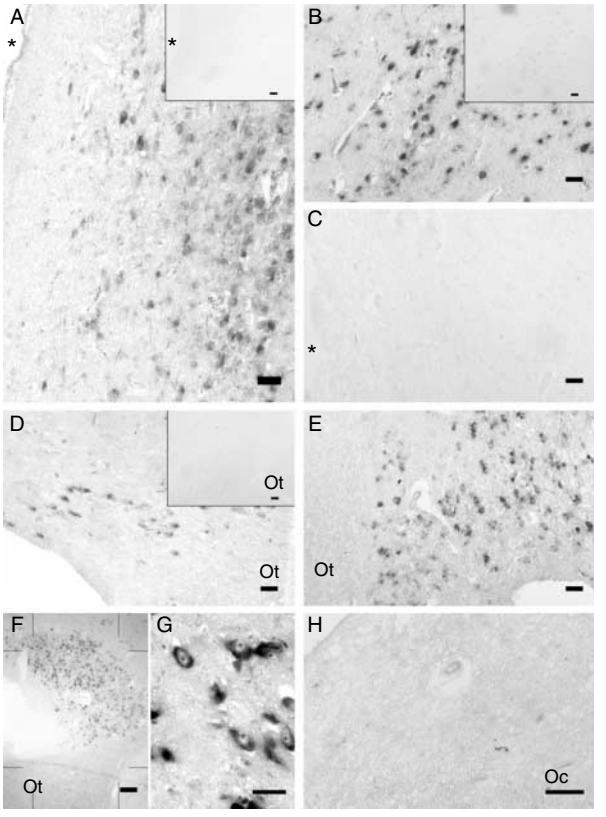

Figure 2 Representative images of *MC4R* mRNA expression in the human hypothalamus. (A) Paraventricular nucleus (sense control in inset) shows intense staining (patient # 2000-007), as does the (B) nucleus basalis of Meynert (sense control in inset) (patient # 1998-127). (C) Infundibular nucleus is devoid of staining (patient # 2000-007). (D) Ventromedial supraoptic nucleus (SON) with (sense control in inset) (patient # 1980-008) shows lower staining intensity than (E) the dorsolateral SON (patient # 1980-008). (F) Low magnification of SON (patient # 1998-161). (G) High magnification of staining of neurons in the dorsolateral SON (patient # 1998-161). (H) No *MC4R*-positive cells in the suprachiasmatic nucleus (patient # 1998-161). Probe concentration 50 nM. Scale bar represents 50 μm. Ot, optic tract; Oc, optic chiasm; *third ventricle.

Co-expression of MC4R *mRNA with GFAP, AVP/OXT, CRH, AgRP, α-MSH, or NPY*

To clarify the identity of smaller *MC4R*-positive cells of the hypothalamus, we examined GFAP and *MC4R* co-expression throughout the hypothalami of three patients (NBB # 1998-161, 2000-090 and 2001-021) and found no GFAP overlap (Fig. 4A).

Co-expression of *MC4R* and CRH was studied in three subjects (a man (NBB # 1998-237), a pre-menopausal woman (NBB #1980-008), and a postmenopausal woman (NBB # 1998-161) (Table 1)). CRH expression in parvocellular neurons has been reported in the human PVN and BST (26), but we did not find co-expression of CRH and *MC4R* in either nucleus (Fig. 4B).

We observed intense *MC4R* expression in the magnocellular neurons of the SON and PVN, which are known to express AVP and OXT (27). Within the SON, we found more magnocellular neurons positive for *MC4R*, with much darker staining, in the dorsolateral compared with the ventromedial part. The size of these neurons indicates that these are neurosecretory neurons, which was confirmed by co-localization with an antibody raised against AVP/OXT. Thus, we found that in the dorsolateral SON, the majority of *MC4R*-positive magnocellular neurons express AVP/OXT, while approximately half of the AVP/OXT-expressing cells are *MC4R* positive (Fig. 4C). Overlap with AVP/OXT-positive neurons in the PVN, however, was much more limited.

AgRP and α-MSH are endogenous ligands of the MC4R, acting as an inverse agonist and an agonist respectively. Innervation of the human PVN by neurons containing these peptides has been established (23, 28). We therefore set out to examine whether these neuropeptides are indeed in close proximity of cells that are positive for *MC4R* mRNA in the PVN. With antibodies directed against the two neuropeptides, we found staining in close juxtaposition of *MC4R*-positive cells in the PVN (Fig. 4D and E), but no co-localization.

NPY, like AgRP and α-MSH, shows a dense immunostaining in the PVN (23). In this study, we found intense NPY staining in the vicinity of *MC4R*-positive neurons in the PVN (Fig. 4F), but no co-localization of *MC4R* and NPY.

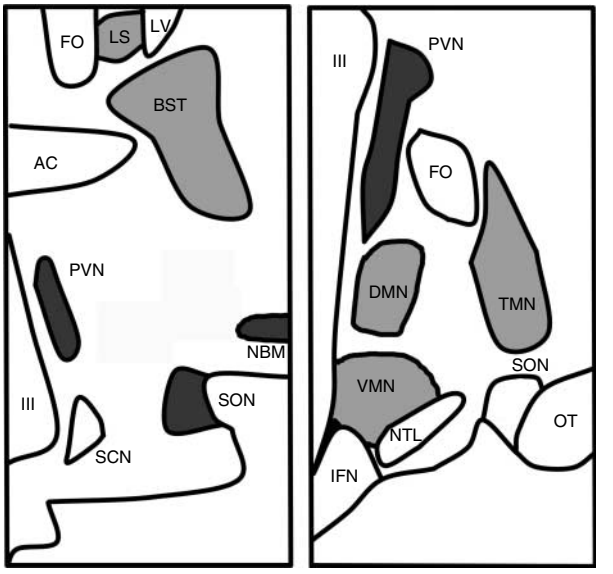

Figure 3 Schematic representation of melanocortin 4 receptor expression in the human hypothalamus. Left panel is at the rostral and right panel at the caudal level of the hypothalamus. Dark gray coloring indicates areas showing intense staining. Light gray areas indicate less intense staining. III, third ventricle; AC, anterior commissure; BST, bed nucleus of the stria terminalis; DMN, dorsomedial hypothalamic nucleus; FO, fornix; IFN, infundibular nucleus; LS, lateral septum; LV, lateral ventricle; NBM, nucleus basalis of Meynert; NTL, nucleus tuberalis lateralis; OC, optic chiasm; OT, optic tract; PVN, paraventricular nucleus; SCN, suprachiasmatic nucleus; SON, supraoptic nucleus; TMN, tuberomammillary nucleus; VMN, ventromedial hypothalamic nucleus.

Table 3 Distribution of *MC4R* mRNA in the human hypothalamus.

Preoptic region	
SONvm	+
SONdm	++
SCN	−
PVN	++
MPO	+
NBM	++
Septum	
LS	+
MS	−
Tuberal region	
VMH	+/−
DMH	+/−
LH	+/−
INF	−
TMN	+
BST	+
NTL	+/−
Mammillary region	
MM	+
MML	+

SONvm, supraoptic nucleus, ventromedial part; SONdm, supraoptic nucleus, dorsolateral part; SCN, suprachiasmatic nucleus; PVN, paraventricular nucleus; MPO, medial preoptic area; NBM, nucleus basalis of Meynert; LS, lateral septal nucleus; MS, medial septal nucleus; VMH, ventromedial nucleus; DMH, dorsomedial hypothalamic nucleus; LH, lateral hypothalamic area; INF, infundibular nucleus; TMN, tuberomammillary hypothalamic nucleus; BST, bed nucleus of the stria terminalis; NTL, nucleus tuberalis lateralis; MM, mammillary nucleus; MML, medial mammillary nucleus, lateral part. −, no staining; +/−, some cells have light staining; +, most cells are positive and show a moderate-strong signal; ++, most to all cells are positive and staining is intense.

Discussion

By examining mRNA expression in postmortem brain material, using LNA-2′-*O*-methyl-RNA-ISH, we are the first to describe the cellular *MC4R* expression and widespread distribution in the human hypothalamus.

Distribution of MC4R *mRNA*

As *MC4R* mutations lead to similar phenotypes in mice and humans, we expected and found the *MC4R* mRNA expression pattern to be similar to that described in rodents (29), although some differences were present. We found high levels of *MC4R* mRNA in the PVN, which is in agreement with *Mc4r* expression in the PVN of rodents (14, 15). Also, the SON was a site of high expression, as reported previously in rats, but not in mice (17). MC4R in the PVN is important for eating behavior, but the function of *MC4R* expression in the SON is unknown at present. *MC4R* was absent in the human IFN and SCN. In mice and rats, however, low expression levels of *Mc4r* were reported in the arcuate nucleus. No *Mc4r* expression was reported in the SCN of mice (15), while in rats either high levels (13) or a complete absence has been reported (14).

We have previously investigated the effects of possible confounders on the expression levels of hypothalamic mRNAs and proteins and found no effects of postmortem delay, fixation duration, age, or sex (30, 31, 32, 33, 34). Although *MC4R* expression levels in this study may have been affected by metabolic factors, premortem illness, as well as cause of death in our studies, as has been shown to be the case with NPY and TRH expression levels (23, 30), it is highly unlikely that the distribution pattern of the MC4R is altered by disease, as this pattern was similar in all subjects included in our study.

Whether the interindividual differences in staining intensity we observed in this study are related to BMI is interesting to contemplate. Also, although casual observation may be interpreted to suggest an inverse relationship between staining intensity and BMI in the four individuals with known BMI, the present experimental design does not allow us to draw any conclusions on the relationship between *MC4R* expression and BMI, especially not in view of possible nonlinear relations between neuropeptide expression and BMI as we described previously (34). To uncover a possible relationship between *MC4R* expression and BMI, a similar experimental setup is required as we have published previously (34), which lies beyond the scope of these studies, but which will be the aim of our future studies.

In the LH, VMN, and DMN, we found lower levels of *MC4R* mRNA compared with the PVN or SON, which is

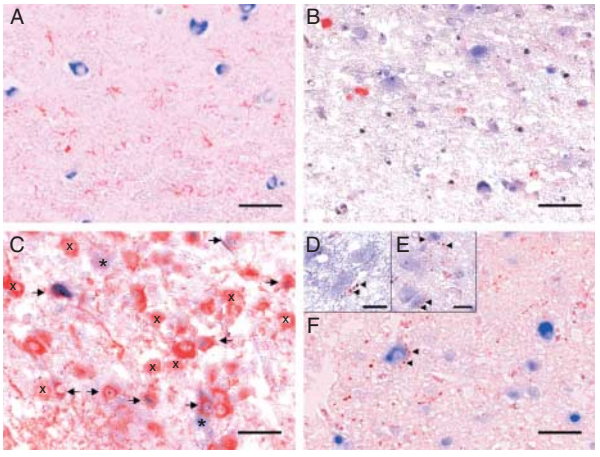

Figure 4 Representative images of *MC4R* mRNA in relation to glial fibrillary acidic protein (GFAP), corticotropin-releasing hormone (CRH), vasopressin/oxytocin (AVP/OXT), agouti-related Protein (AgRP), α-melanocyte stimulating hormone (α-MSH), and neuropeptide Y (NPY) in the human hypothalamus. (A) GFAP (red) spindles and *MC4R*-positive neurons (blue) do not show overlap (patient # 1998-161, bed nucleus of the stria terminalis). (B) CRH- (red cells) and *MC4R*-expression (blue) do not overlap (image of the paraventricular nucleus (PVN)) (patient # 2000-007). (C) AVP/OXT (red) and *MC4R* (blue) in supraoptic nucleus do overlap (arrows), or are only positive for AVP/OXT (*), or *MC4R* (x) (patient # 2000-090). (D) AgRP (red) (patient # 2000-090, PVN), (E) α-MSH (red) (patient # 1998-161, PVN), and (F) NPY (red) can be found in close proximity (arrowheads) to *MC4R*-positive neurons (blue) (patient # 2001-021, PVN). Scale bars represent 50 μm.

consistent with findings in rodents (17). These nuclei are important sites for the integration of energy, glucose, and insulin homeostatic signals, and these systems become unbalanced when MC4R function is reduced (10).

The human NBM has been extensively studied in view of its role in aging and Alzheimer's disease (35). The function of MC4Rs in the NBM is currently unknown, but as we observed the highest expression levels within each patient in this particular nucleus, this will be an interesting topic for future research.

In conclusion, we found the distribution of *MC4R* mRNA-expressing cells between human, rats, and mice to be largely in agreement. This observation may assist in translating the large body of rodent data on MC4R to humans.

MC4R and co-localization with other neuropeptides

MC4R expression has previously been reported in primary rat astrocyte cultures derived from cortex (36), arcuate nucleus, and VMH (37). We used GFAP to identify astrocytes in the human hypothalamus but found no overlap with *MC4R* mRNA expression (Fig. 4). This may indicate a species difference or reflect an adaptation of cultured astrocytes to an *ex vivo* environment in the studies published earlier. The neuropeptidergic content of smaller *MC4R*-positive cells, such as those in Fig. 4A, requires further co-localization studies. Whether these cells could represent GFAP-negative astrocytes, oligodendrocytes, or perhaps microglia remains unclear.

The SON is an important source for circulating AVP (27) and showed high *MC4R* mRNA expression. AVP release is driven by serum osmolality, and inappropriately low plasma levels as seen in central diabetes insipidus lead to a hyperosmolar state with polydipsia and polyuria (38). Interestingly, the $Mc4r^{K314X}$ mutation in the rat described by Mul *et al.* (39) induces polydipsia. In view of the currently reported co-localization of AVP and *MC4R*, it is tempting to hypothesize that a deficient AVP release in the $Mc4r^{K314X}$ rat may be involved in the pathogenesis of the reported polydipsia.

CRH is mainly expressed in the parvocellular cells in hypothalamic nuclei, a cell type in which we also observed *MC4R* mRNA. Although AgRP has been reported in close proximity to a small percentage of CRH neurons in humans (26), and part of the CRH neurons in the rat PVN express *Mc4r* (40), we did not observe co-localization of *MC4R* and CRH in the human PVN. Expression levels of CRH, but not its neuroanatomical distribution, are influenced by gender and age (22). In these studies we have investigated both males and females varying in age, but in none of these subjects did we observe CRH and *MC4R* co-localization. Therefore, these factors are unlikely to explain the lack of co-localization. Other candidates including TRH, somatostatin, and GHRH remain to be investigated.

Innervation by α-MSH, AgRP, and NPY of TRH neurons has been established in the human PVN (28). Although most TRH-positive cells in the PVN are parvocellular, TRH expression in a small number of magnocellular neurons has been described as well (41). Although TRH immunostaining has been reported earlier in picric acid/glutaraldehyde-fixed vibratome sections, it was unsuccessful in formalin-fixed material such as we used (41) and could therefore not be incorporated in this study. In view of the current observation of extensive innervation of *MC4R*-positive neurons by α-MSH, AgRP, and NPY fibers, co-expression of TRH in these magnocellular neurons is highly likely but remains to be investigated using different tissue treatments.

MC4R innervation by α-MSH, AgRP, and NPY

Classically, MC4R is activated by its N-terminus (basal activity) (42) and further stimulated by α-MSH. These activations of the receptor can be undone by the binding of AgRP (2). In line with this concept, we found terminals containing the endogenous binding partners of the MC4R, i.e. α-MSH and AgRP, in close proximity to *MC4R*-expressing neurons in the PVN. This strongly suggests that the functional neuroanatomy of the melanocortin signaling system in humans is similar to that of rodents.

POMC neurons that produce α-MSH and project to the PVN express Y-receptors that enable NPY to indirectly affect MC4R activity through the production and/or release of α-MSH by POMC neurons. However, NPY innervation directly onto the PVN in humans (43), as well as low levels of Y1- and higher levels of Y2-receptor binding sites, have been described in the PVN (44). In addition, Y5 receptor mRNA is expressed in magnocellular neurons of the PVN and SON (45), as well as in other nuclei in which we found *MC4R* to be expressed. In this study, we found NPY terminals in close proximity to *MC4R*-expressing neurons in the PVN (Fig. 4F). In mice, green fluorescent protein under the *Mc4r*-promoter Y1-receptor and GFP co-localization was found in subnuclei of the PVN (46). Using the same mouse model, a strong inhibitory effect of NPY on MC4R-positive cells was measured by electrophysiology (47). NPY receptors inhibit adenylyl cyclase via activation of G_i or G_o proteins (48, 49, 50), thus opposing the G_as-activation of adenylate cyclases that occurs due to MC4R stimulation. These findings could provide an additional pathway for NPY to affect melanocortin signaling, not only through POMC neurons but also directly affecting *MC4R*-positive neurons. Whether this regulatory system is indeed in place in the human brain is currently unknown.

Conclusions and future directions

This study provides the anatomic distribution of *MC4R* mRNA in the human hypothalamus for the first time and starts to unravel the identity of *MC4R*-expressing neurons. Profiling the neurons that express MC4R can provide us with a deeper understanding of the mechanisms underlying the development of obesity both in patients with and without MC4R signaling defects and of other symptoms associated with MC4R mutations.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

E Fliers, A Alkemade, J E Siljee, and D F Swaab were involved in the study concept and design. J E Siljee and U A Unmehopa were involved in acquisition of data. J E Siljee, E Fliers, and U A Unmehopa were involved in analysis and interpretation of data. J E Siljee and A Alkemade were involved in drafting of the manuscript. A Alkemade, E Fliers, A Kalsbeek, and D F Swaab were involved in critical revision of the manuscript for important intellectual content. U A Unmehopa, J E Siljee and A Alkemade were involved in administrative, technical, and material support. A Alkemade was involved in study supervision.

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