

Network: Obesity and Related Disorders**Project: MC4R Signalling within the Hypothalamic Network of Weight Regulation**

Heike Biebermann – Charité Campus Virchow Klinikum (CVK), Berlin - heike.biebermann@charite.de

Introduction

Within the last few years several genes were described to play a role in hypothalamic energy homeostasis in rodents. These genes are expressed in the leptin-melanocortin pathway of hypothalamic regulation of food intake and energy expenditure. Mutations in the human orthologue genes were found as rare causes of monogenetic obesity, e.g. leptin-, leptin-receptor-, proopiomelanocortin-, pro-hormone convertase- and melanocortin-receptor genes. So far the highest frequency of mutations were found in the melanocortin 4 receptor (MC4R) gene occurring in 3 - 5 % of morbidly obese study populations. The MC4R belongs to the large superfamily of G-protein coupled receptors (GPCR) sharing the common structural feature of a seven transmembrane spanning protein. The MC4R is activated by proopiomelanocortin (POMC) derived peptides alpha-MSH and beta-MSH and is antagonized by agouti-related peptide (AGRP). The activated receptor couples to the G_s/adenylyl cyclase system which leads to decreased food intake and increased energy expenditure. Complete loss of POMC-derived peptides results in obesity, red hair and adrenal insufficiency in the affected patients (1). Targeted deletion of the MC4R resulted in severe non-syndromic obesity in homozygous mice while heterozygous mice were shown to have intermediate body weight increases with a more pronounced effect in female animals. These data clearly argued for a gene-dosage effect of the MC4R in weight homeostasis, which contrasts to known genotype-phenotype correlations of so far identified mutations in the GPCR gene family. However, in accordance with mouse MC4R knock-out findings most human mutations were found in heterozygosity. The resulting loss-of-function of the mutant receptors implicates a same gene dosage effect on human weight maintenance as in rodents. Moreover, partial loss-of-function mutations were described resulting in autosomal recessive inheritance of obesity in the affected families with intermediate phenotypes in heterozygous mutation carriers and severe obesity in homozygous patients. Based on these data, the molecular mechanism of the clinical occurrence of obesity due to heterozygous MC4R mutations is most likely a dosage effect and -within this line of argumentation- a dominant negative effect was so far excluded for two loss-of-function mutations. Recently we could show for a heterozygous MC4R mutation, D90N, located in the second transmembrane domain, that this receptor variant had a dominant-negative effect on wild-type receptor function. To understand the molecular mechanism of this effect we investigated MC4R dimerization and could show for the first time that the MC4R is able to form receptor oligomers (2).

Results/Project Status**Complete and partial loss of POMC function**

After the identification of the first two patients with complete loss of function mutations in the POMC gene we could identify three more patients based on the clinical appearance of severe early onset obesity, red hair and adrenal insufficiency (3). The body weights of the parents of the affected patients who are heterozygous POMC mutation carriers are the upper normal range or slightly overweight indicating a gene dosage dependence.

Mutations in the MC4R gene

In a cohort of 300 early onset obese patients from the Berlin Children Hospital we identified 17 variations. In 9 patients we identified the known polymorphisms I251L and V103I. In seven patients we found heterozygous missense mutations.

Furthermore in one family with two extremely obese patients we identified a homozygous mutation (C271R). For functional characterization we cloned the mutated receptor in an eukaryotic expression vector, transfected transiently COS-7 cells and investigated the mutated receptor in view of cell surface expression, binding of the ligand and signal transduction properties after stimulation with the endogenous ligands α - and β -MSH and the highly potent ligand NDP- α -MSH.

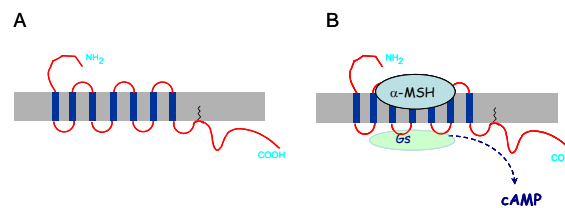


Fig 1: Schematical structure of the MC4R and its ligand induced activation.

(A) The structure of the MC4R consists of seven transmembrane domains that are connected by three extracellular and three intracellular loops. The N-terminus is located extracellularly and the C-terminus intracellularly. (B) Activation of the receptor by its natural ligand α -MSH results in activation of the G_s/adenylyl cyclase pathway leading to enhancement of intracellular second messenger cAMP.

Functional characterization of the four mutations (D90N, C271R, N274S, A175T) revealed a partial loss of function for three mutations. The D90N is a complete loss of function mutation that could be identified to cause a dominant-negative effect. Moreover we performed a detailed analysis of the homozygous mutation, C271R, located in the third extracellular loop of the receptor. To gain insight into the molecular mechanism of the inactivating mutation C271R the mutation was further characterized by systematic site-directed mutagenesis. Our data strongly support a new mechanism in which the receptor malfunction is induced by formation of a functionally disastrous disulfide bridge between Cys277 and Cys279 (Fig.2). Mutational and chemical disruption of this improper disulfide bond was able to restore normal receptor potency. This knowledge may be useful to develop new therapeutic strategy to treat this specific form of hereditary obesity (4).

In collaboration with Prof. Hebebrand and Dr. Hinney we functionally characterized 8 new MC4R mutations identified in the KORA (Kooperative Gesundheitsforschung in der Region Augsburg) collective. Signal transduction properties of all investigated mutations revealed functional characteristics comparable to the wild-type receptor for all mutations despite one (H158R) that revealed basal constitutive activation. Furthermore we investigated 8 MC4R mutations identified in obese patients. So far 5 mutations were analyzed. From these mutations 3 were complete loss of function mutations, one partial loss of function mutations and one have functional properties comparable to the wild-type receptor

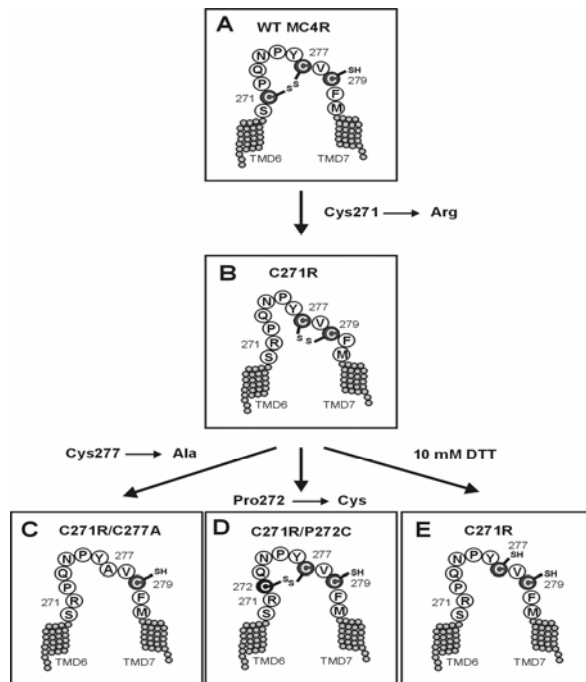


Fig 2: Proposed mechanisms of functional reconstitution of C271R.

A schematic model of the third extracellular loop (EL3) and the flanking transmembrane domains is shown. In the WT MC4R Cys271 and Cys279 are connected via a disulfide bridge (A). Mutation of Cys271 to Arg induces the formation of a disulfide bond between Cys277 and Cys279 which leads to an improperly folded EL3 (B). Destruction of the improper disulfide bridge by mutation of Cys277 to Ala (C), introduction of an additional Cys residue at position 272 (D) or treatment with 10 mM DTT (E) can partially restore the function of C271R.

MC4R oligomerization

A prerequisite to develop a highly potent and selective hypothalamic MC4R agonist is a more detailed understanding of receptor function. One successful tool to learn more about structure-function relationships of a specific receptor is the characterization of naturally occurring mutations. In the MC4R a large number of mutations have been identified in obese patients. Interestingly, these inactivating mutations are mostly heterozygous. A dominant negative effect has been ruled out for some mutations. However, the investigation of one heterozygous MC4R mutation, D90N, in transmembrane domain 2 has revealed a dominant negative effect on wild-type receptor function. Further studies have shown that dimerization of mutant and wild-type receptor is the underlying molecular mechanism for the dominant negative effect (2). To understand how the MC4R dimerizes we investigated the role of extracellular cysteine residues for receptor dimerization because extracellular cysteine residues could be responsible for dimerization of for example for dimerization of the calcium sensing receptor. We performed single or multi cysteine exchanges and investigated receptor dimerization. We could show that neither single nor multi extracellular cysteine residues play a functional role in receptor dimerization. We concluded from these findings that for MC4R dimerization structures of the transmembrane-spanning helices are more likely to be involved.

Rescue of functional inactive MC4R mutations

We investigated two mouse MC4R mutations: I194F located at the interface of the second extracellular loop/fifth

transmembrane domain and Y302C in the seventh transmembrane domain. Stimulation of Y302C with natural and highly potent MC4R ligands did not result in activation of the Gs/adenylyl cyclase pathway. For the I194F mutation we could show that stimulation with α - and β -MSH resulted in cAMP formation, however significant more ligand was needed to stimulate the mutated receptor compared to the wild-type receptor. Stimulation with the artificial ligand NDP- α -MSH was comparable to that of the wild-type receptor indicating that specific MC4R mutations could be rescued with artificial ligands. We then identified a heterozygous human MC4R mutation, S127L, for which we were able to demonstrate a partial loss of function after stimulation with natural ligands but rescue of function after stimulation with NDP- α -MSH (Fig. 3)

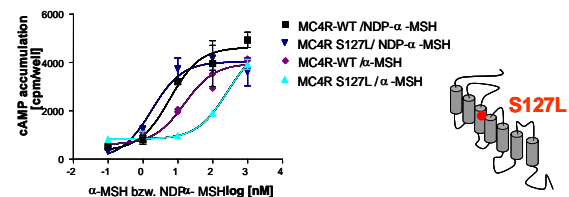


Fig 3: Functional characterization of wild-type and S127L mutant MC4R.

COS-7 cells were transiently transfected with wild-type and mutant MC4R and intracellular cAMP accumulation was determined after stimulation with increasing concentrations of α -MSH and NDP- α -MSH.

Outlook

Functional characterization of naturally occurring MC4R is an in value opportunity to gain insight into functional relevant regions of the receptor. Functional important receptor regions are highly conserved throughout species. We therefore started to investigate the MC4R in over 30 different species including mammals, birds and fishes being slim or fat under natural conditions. These findings will teach us more about the fine-structure of the MC4R and more detailed MC4R function.

One crucial feature of MC4R function is MC4R oligomerization. Functional characterization that mimic the heterozygous state of MC4R mutations (co-transfection of wild-type and mutant alleles) will help to point to receptor regions that are involved in dimerization. However, if several amino acid residues work in concert to form an extended binding interface, the contribution of specific amino acid residues may be difficult to determine. The ongoing identification of naturally occurring MC4R mutations may help to detect receptor structures that are involved in receptor-receptor interaction. Additionally, heterodimerization of the MC4R with tissue-specific interaction partners may be instrumental for the future design of selective MC4R ligands for anti-obesity treatment.

Lit.: 1. Krude H et al. Severe early-onset obesity, adrenal insufficiency and red hair pigmentation caused by POMC mutations in humans. *Nature Genetics*; **19**:155-157. (1998). 2. Biebermann H et al., et al. Autosomal dominant mode of inheritance of a MC4R mutation in a patient with severe early-onset obesity is due to a dominant negative effect caused by receptor dimerization. *Diabetes*, **52**:2984-2988 (2003) 3. Krude H et al. Obesity due to proopiomelanocortin deficiency: Three new cases and treatment trial with thyroid hormone and ACTH 4-10; *J Clin Endocrinol Metab*, **88**:4633-40. (2003) 4. Tarnow P et al., Mutational induced disulfide bond formation within the third extracellular loop causes melanocortin 4 receptor inactivation in patients with obesity, *J Biol Chem*, **278**:48666-48673 (2003).