FEBS Letters 526 (2002) 124–128 FEBS 26373

MIZIP, a highly conserved, vertebrate specific melanin-concentrating hormone receptor 1 interacting zinc-finger protein[1]

Dietmar Bächner*, Hans-Jürgen Kreienkamp, Dietmar Richter

Institut für Zellbiochemie und klinische Neurobiologie, Universität Hamburg, Martinistrasse 52, 20246 Hamburg, Germany

Received 4 July 2002; revised 13 July 2002; accepted 13 July 2002

First published online 30 July 2002

Edited by Gianni Cesareni

Abstract Using the yeast-two-hybrid system a novel protein was identified from human brain that interacts with the C-terminus of melanin-concentrating hormone receptor 1 (MCH-R1). This protein, characterized by a Myeloid translocation protein 8, Nervy, DEAF1 proteins (MYND) zinc-finger domain, is termed MCH-R1-interacting zinc-finger protein, MIZIP. It is fully conserved in man, rat, mouse and highly conserved in *Xenopus* and zebrafish, but not detectable in invertebrates. MIZIP gene organization in human (six exons on chromosome 9q34.3) and mouse is highly conserved, yet in rodents an additional exon is generated giving rise to alternatively spliced mRNAs. MIZIP is expressed in brain, testis and stomach, where expression of MCH and MCH-R1 was previously reported. MIZIP interaction with MCH-R1 was verified by overlay and pull-down assays as well as by co-transfection experiments in human embryonic kidney-293 cells. MIZIP is cytoplasmically localized but gets recruted to the plasma membrane when cells are co-transfected with MCH-R1 supporting the notion that MIZIP is involved in the function of MCH-R1.

Key words: Yeast-two-hybrid; MYND-zinc finger; G protein-coupled receptor interacting protein; Food-intake behavior

1. Introduction

In mammals the nonadeca neuropeptide melanin-concentrating hormone (MCH) is known to be involved in the control of food intake and energy metabolism [1]. Recently, we [2] and several other groups independently identified the orphan G protein-coupled receptor (GPCR) somatostatin-like-clone-1 as an MCH receptor (MCH-R1) (reviewed in [3]). MCH binds to MCH-R1 with nanomolar affinity and activates Gi/o and Gq coupled signal transduction pathways [2,4]. Recently, evidence has emerged that the functions of GPCRs involving desensitization, internalization, subcellular localization, and specific signaling capacities are regulated via multiple interactions with additional proteins mainly via the C-terminus of these receptors [5,6]. Using the yeast-two-hybrid system we report here the identification of a novel protein, termed MIZIP, fully conserved in human, mouse and rat, that specifically interacts with the C-terminus of MCH-R1, an interaction that has been verified by overlay, pull-down and co-transfection experiments. The data suggest that MIZIP may be involved as a regulatory molecule in MCH-R1 signaling.

2. Materials and methods

2.1. Yeast-two-hybrid screen

A cDNA fragment (nucleotides 915–1085) coding for the rat MCH-R1$_{299–353}$ [7] was cloned into the yeast bait vector pAS2 (BD Biosciences, Palo Alto, CA, USA). For transformation of the yeast reporter strain, screening of a human brain cDNA-library in the yeast fish vector pACTII and the β-galactosidase filter lift assay see [8].

2.2. Overlay assays

Full-length human MIZIP-cDNA was cloned into the glutathione *S*-transferase (GST) vector pGEX-4T2 (Amersham, Uppsala, Sweden) and a rat MCH-R1-cDNA fragment (nucleotides 915–1085) into the dehydrofolate reductase (DHFR)-HIS vector pQE40 (Qiagen, Hilden, Germany). Fusion proteins were expressed in *Escherichia coli* strain BL-21 (Stratagene, Amsterdam, The Netherlands) and purified on glutathion–Sepharose (Amersham) or Ni-NTA agarose (Qiagen) following the instructions of the manufacturers. For overlay assay 20 μg GST–MIZIP and GST control proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto a nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany). The membrane was stained (10 min) with 1% Ponceau-S, blocked in TBS-T (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20) supplemented with 10% milk-powder (3 h, 4°C) and incubated with 2 μg/ml DHFR-HIS–MCH-R1 fusion protein for 14 h (4°C) under constant shaking. The bound proteins were detected using mouse anti-HIS or rabbit anti-MCH-R1, kindly provided by Dr. Guillaume Hervieu, Smithkline-Beecham, Essex, UK [9], as primary antisera and secondary antibodies coupled to alkaline-phosphatase or horseradish peroxidase.

2.3. GST pull-down assays

20 μg GST control and GST-MIZIP fusion proteins were coupled to glutathion–agarose and incubated in 50 mM Tris–HCl, pH 7.4, 0.1% Triton X-100 supplemented with 2 μg/ml purified DHFR-HIS–MCH-R1 fusion protein (3 h, 4°C) under continuous shaking. After washing bound proteins were eluted by boiling in SDS sample buffer and analyzed by Western blotting using the antisera described for the overlay assay.

*Corresponding author. Fax: (49)-40-42803 4541.
E-mail address: baechner@uke.uni-hamburg.de (D. Bächner).

[1] Nucleotide sequence data reported are available in the EMBL database under accession numbers AJ298882 (human MIZIP), AJ409150 (murine MIZIPa), AJ409151 (murine MIZIPb), AJ488144 (rat MIZIP), and AJ488145 (*Xenopus laevis* MIZIP).

Abbreviations: DHFR, dehydrofolate reductase; EST, expressed sequence tag; GST, glutathione S-transferase; HEK, human embryonic kidney; MCH, melanin-concentrating hormone; MYND, zinc-finger domains of **M**yeloid translocation protein 8, **N**ervy, **D**EAF1 proteins; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; RACE, rapid amplification of cDNA ends

0014-5793/02/$22.00
PII: S0014-5793(02)03115-0

2.4. Expression in human embryonic kidney (HEK)293 cells and immunocytochemistry

The cDNAs for rat MCH-R1, cloned into a modified pcDNA3 plasmid [10] (Invitrogen, Karlsruhe, Germany) providing an N-terminal T7-epitope tag, and mouse MIZIP, cloned into pcDNA6-myc-his (Invitrogen) providing an C-terminal myc-his-epitope tag, were expressed in HEK293 cells by transient transfection using the calcium-phosphate method as described [8]. For immunocytochemical analysis see [8].

2.5. Identification of the cDNAs encoding murine, rat, Xenopus laevis and zebrafish MIZIP

The cDNA sequence of human MIZIP was used to screen (NCBI-BLAST) public expressed sequence tag (EST)-databases for homologous sequences. Overlapping ESTs were identified, covering most of the coding region of murine MIZIP, PCR primers were used to isolate the cDNA of murine MIZIPa out of murine embryonic and adult brain cDNAs. Two additional murine ESTs with partial overlapping sequence were identified and used to generate PCR-primers that allowed the isolation of murine variant MIZIPb. The same approach was used to identify the cDNA coding for rat MIZIP. To obtain full-length cDNA sequence of *X. laevis* MIZIP an 3′-RACE (rapid amplification of cDNA ends) kit (Roche Diagnostics, Mannheim, Germany) and *X. laevis* oocyte cDNA was used. The putative sequence of zebrafish MIZIP was obtained by alignment of overlapping EST sequences.

2.6. RNA-expression analysis

Northern blots (human brain MTN blot IV, mouse MTN blot) and the multiple tissue array dot blot human MTE were hybridized using ExpressHyb solution supplemented with a α-[^{32}P]dCTP-labeled cDNA probe of human/mouse MIZIP as outlined (BD Biosciences). RNA in situ hybridization was performed as described [11]. Antisense and sense RNA probes were generated by in vitro transcription using α-[^{35}S]UTP and full-length murine MIZIP cDNA.

3. Results

To identify proteins that associate with the C-terminus of MCH-R1 a human brain cDNA library was screened with rat MCH-R1$_{299–353}$ as a bait using a yeast-two-hybrid assay. From a total of 2.7×10^6 screened clones 20 clones were positive after selection of which 14 were identified by sequencing. Five clones were identical in sequence (nucleotides 1–1378 of EMBL acc. no. AJ298882) encoding a novel protein. Amino acid sequence comparison (NCBI-BLAST) revealed an evolutionary conserved zinc-finger motif in the C-terminal region of the identified protein (Fig. 1A) that has a high degree of amino acid identity to the zinc-finger domains of **My**eloid translocation protein 8, **N**ervy, **D**EAF1 proteins (MYND)-domain of proteins such as the human tumor suppressor BLu [12], human proto-oncogene Myeloid translocation protein 8, MTG8 [13], mouse deformed epidermal autoregulatory factor 1, Deaf1 [14], and an *Arabidopsis* protein with unknown function, F1N21.16 (EMBL acc. no. AAG002130) (Fig. 1B). Therefore we designated this protein as MCH-R1 interacting zinc-finger protein, MIZIP. The MYND-domain is a cysteine repeat motif first discovered as protein–protein interaction domain of nuclear proto-oncogenes involved in the regulation of gene transcription [13,15], yet the precise functions of the more than 100 identified MYND-domain containing proteins are unknown. Analysis of the MIZIP amino acid sequence with PROSITE motif search predicted multiple putative phosphorylation sites (Fig. 1A) suggestive for a regula-

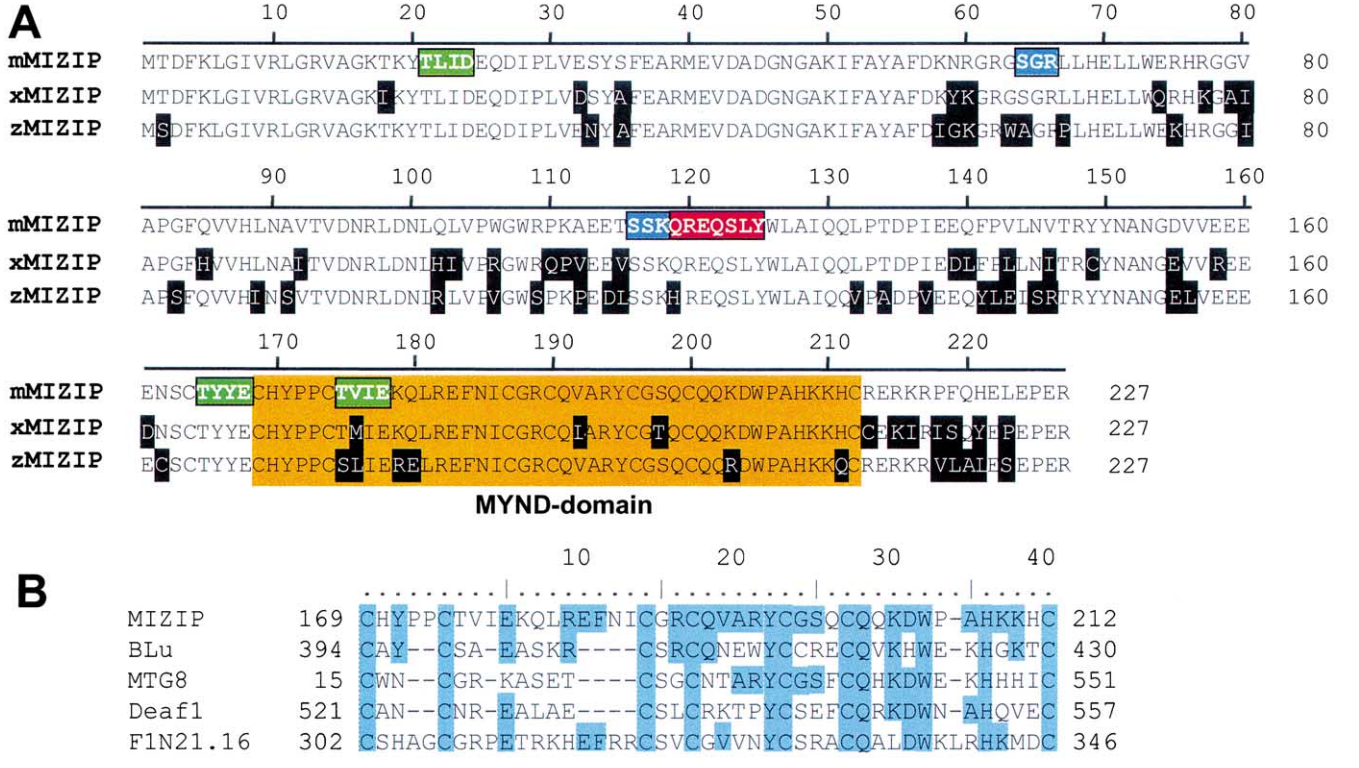

Fig. 1. Amino acid sequence comparison of vertebrate MIZIP orthologues. A: The amino acid sequences of mammalian (m: human, mouse, rat), *Xenopus* (x), and zebrafish (z) MIZIP were aligned using the Clustal algorithm of Megalign (Lasergene). Residues that differ from the mammalian sequence are highlighted as black boxes. The amino acid sequence of MIZIP is identical to that of Q96E35 (TrEMBL), a protein recently predicted through GenomeScan and full-length EST clone sequencing efforts of the NIH Mammalian Gene Collection program. The conserved MYND zinc-finger domains are indicated in orange and putative phosphorylation sites detected by PROSITE motif search in green (CK2), blue (PKC) and red (Tyr-kinase). B: Comparison of the putative MYND zinc-finger domains of MIZIP, BLu, MTG8, Deaf1 and F1N21.16. Identical amino acids are shown in blue.

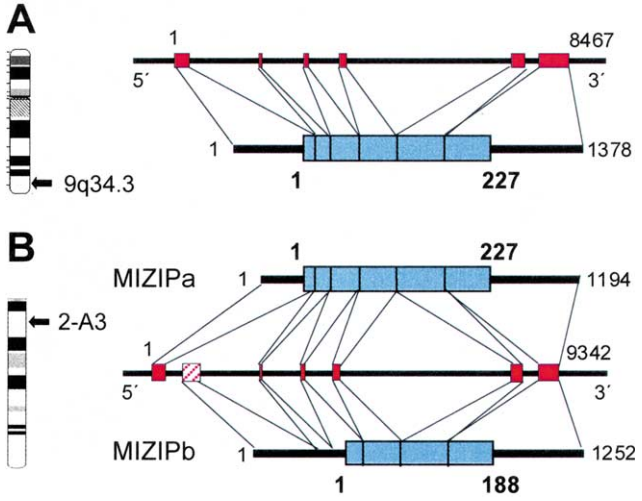

Fig. 2. Gene organization of human and murine MIZIP. A: Human MIZIP gene was determined using the genomic sequence NT_023929.4, localized to chromosome 9q34.3 (left). B: Murine MIZIP gene was determined using the genomic sequence RP23–104G5, localized to mouse chromosome 2-A3 (left). Exons are indicated as red boxes, size of the boxes and distances inbetween correlate to exon and intron length in base pairs; numbers on top refer to gene length in base pairs; the spliced mRNA is indicated below, including open reading frames as blue boxes; the numbers refer to cDNA length in base pairs and the bold numbers to amino acids of the encoded protein, respectively. The hatched box in B indicates the alternative exon 1b specific to mouse MIZIP gene.

tion of MIZIP function by kinases like casein kinase 2 (CKII) or protein kinase C (PKC).

MIZIP orthologues in mouse, rat, *Xenopus* and zebrafish were obtained by EST-database analysis, PCR and 3′-RACE–PCR. Comparison of the deduced amino acid sequences revealed 100% identity between the mammalian MIZIP orthologues and 84% identity between mammalian and *X. laevis*, and 81% between mammalian and zebrafish orthologues, respectively (Fig. 1A). No orthologues were detected in invertebrates suggesting that MIZIP is a highly conserved protein with specific functions in vertebrates as are MCH and MCH-R1.

The MIZIP gene from man and mouse consists of six exons and can be localized on chromosome 9q34.3 (man) and on 2-A3 (mouse). The gene organization is highly conserved between both species in respect to exon and intron sizes, while the splice sites are identical (Fig. 2). An additional exon, 1b, is specific to mouse, but absent in the human counterpart (hatched box in Fig. 2B). It is used to generate an alternative mRNA. Exon 1b lacks any start codon, but an alternative open reading frame is created, starting with an in frame ATG within exon 3, encoding a truncated MIZIP protein, MIZIPb, that lacks the 39 N-terminal amino acid residues of full-length MIZIP (Fig. 2B).

To verify MIZIP/MCH-R1 interaction overlay and pull-down assays using recombinant expressed fusion proteins were performed. In an overlay assay, where GST-fusion protein containing MIZIP was blotted and incubated with a MCH-R1 fusion protein, a strong interaction was detected (arrowhead in Fig. 3A) but not with GST as a control. The specificity of this interaction was further confirmed in a GST pull-down assay. There, immunoreactivity of HIS–MCH-R1 is specifically detected in the pellet of GST–MIZIP but not in GST control (Fig. 3B). Both in vitro assays indicate a direct and specific binding of MIZIP to the C-terminus of MCH-R1.

Further evidence supporting the interaction between MIZIP and MCH-R1 was obtained when analyzing co-transfected HEK293 cells. Transfection with MIZIP alone resulted in a cytoplasmic localization (Fig. 3Ca), whereas transfected MCH-R1 is located at the plasma membrane (Fig. 3Cb). In contrast, when HEK cells were co-transfected with MIZIP and MCH-R1, both proteins co-localize at the plasma membrane (arrows in Fig. 3Cc–e), suggestive for a recruitment of

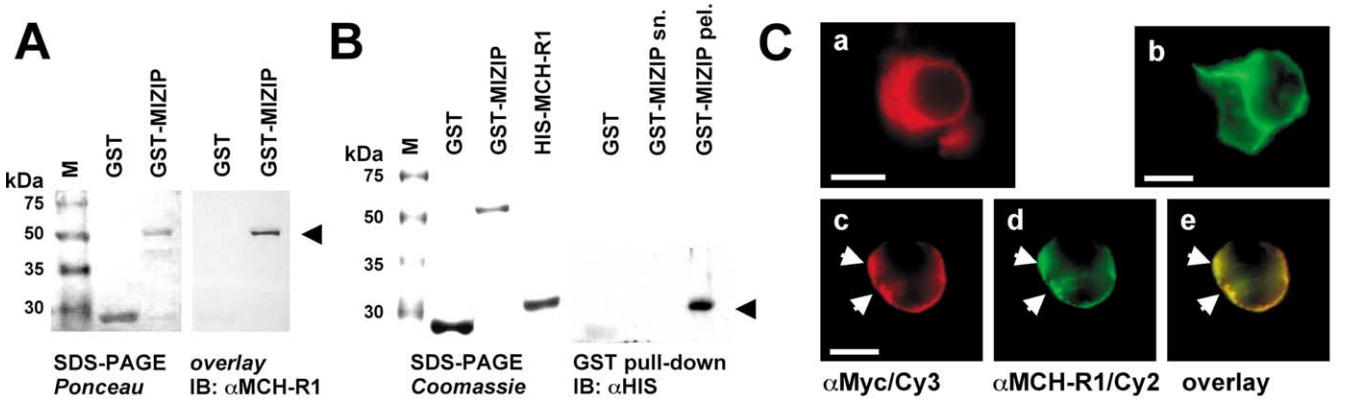

Fig. 3. In vitro interaction of the C-terminus of MCH-R1 with MIZIP. A: Overlay assay. GST–MIZIP fusion proteins and GST-control proteins were run on SDS–PAGE, blotted onto nitrocellulose, stained with Ponceau red (left panel) and subsequently processed for overlay assay with a HIS-DHFR–MCH-R1 fusion protein. Specifically bound protein (arrowhead) was detected with anti-MCH-R1 antiserum. B: Pull-down assay. For control, aliquots of GST–MIZIP fusion protein, GST-control protein and of HIS-DHFR–MCH-R1 were analyzed by SDS–PAGE and stained with Coomassie brilliant blue (left panel). GST-fusion proteins were immobilized on glutathion–Sepharose and incubated with purified HIS-DHFR–MCH-R1. After washing, specifically bound protein (arrowhead) was detected by Western blotting using anti-HIS antiserum. IB, immunoblot; M, rainbow protein standard; pel, pellet; sn, supernatant. C: Localization in co-transfected HEK293 cells. HEK cells were transfected with cDNA constructs encoding C-terminal myc epitope-tagged murine MIZIP (a,c–e) and N-terminal T7 epitope-tagged rat MCH-R1 (b–e), grown on glass coverslips and analyzed by immunocytochemistry using rabbit anti-MCH-R1 antiserum and goat anti-rabbit antiserum coupled to Cy3, or mouse anti-myc epitope antiserum and goat anti-mouse antiserum coupled to Cy2, respectively. In co-transfected HEK cells MIZIP and MCH-R1 are co-localized at the same structures of the plasma membrane (arrows in c–e). Bars, 10 μm.

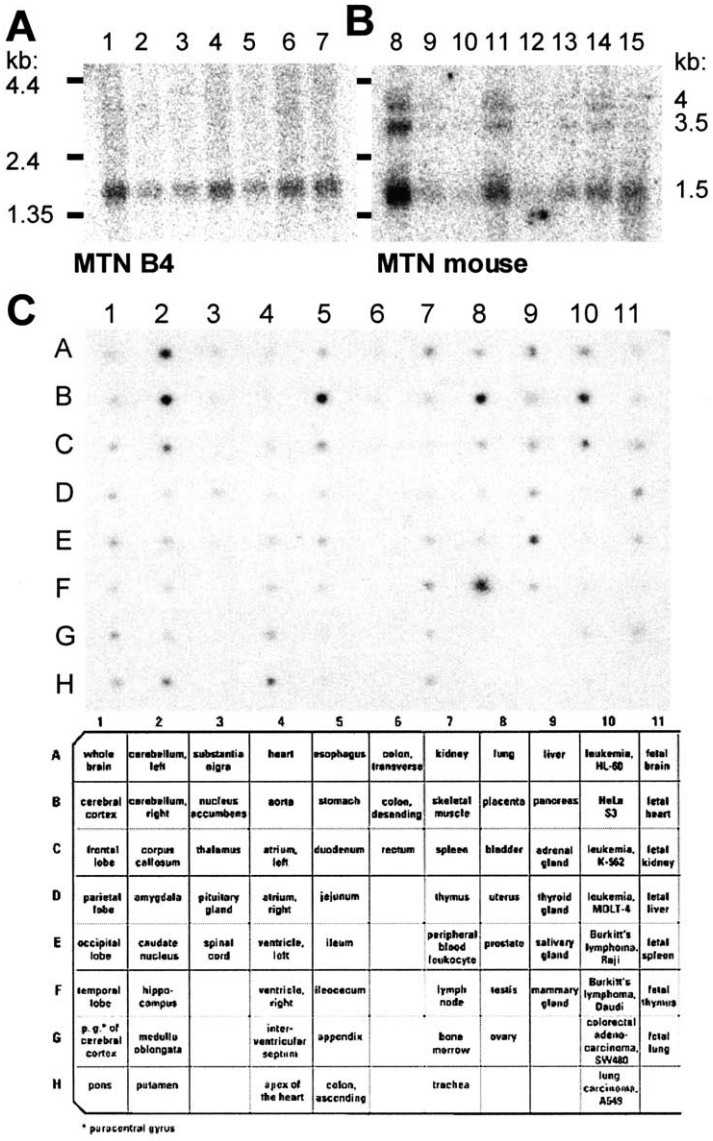

Fig. 4. Northern hybridization analysis of MIZIP in human and murine tissues. Human multiple tissue Northern blot MTN brain 4 (A), mouse MTN (B), and human MTE array (C) were hybridized with a α-^{32}P-labeled human or mouse MIZIP cDNA probe. 1, Thalamus; 2, substantia nigra; 3, brain; 4, hippocampus; 5, corpus callosum; 6, caudate nucleus; 7, amygdala; 8, testis; 9, kidney; 10, skeletal muscle; 11, liver; 12, lung; 13, spleen; 14, brain; 15, heart.

MIZIP to the plasma membrane by interacting with MCH-R1.

To get more information about the putative MIZIP function, mRNA expression analysis using Northern blot and RNA in situ hybridization techniques were performed. Northern blots revealed high expression of a single 1.5 kb mRNA in all areas of human brain, especially in the cerebellum as well as in non-neuronal tissues (heart, liver, skeletal muscle, kidney, testis, placenta, stomach; Fig. 4A,C). Similar expression patterns have been observed for MCH-R1 and MCH [9,16–18].

In the mouse additional mRNAs of 3.5 and 4 kb in size are detectable although no cell-type-specific splicing has been detected (Fig. 4B), suggestive for a constitutive expression of the murine MIZIP variants. RNA in situ hybridization to adult murine brain sections revealed MIZIP expression in all brain areas (Fig. 5a). Enhanced expression is restricted to the granular layer of the cerebellum (Fig. 5c) and to the dentate gyrus (arrows in Fig. 5d) and ammons horn of the hippocampus formation (arrowheads in Fig. 5d). A similar expression pattern was described for rat and mouse MCH-R1 [9,17], suggestive for a co-expression of both genes within the same cell-types of the brain. Hybridization with a sense control probe revealed only background grain distribution (Fig. 5b).

The main function of MCH/MCH-R1 in higher vertebrates seems to be the regulation of appetite behavior and energy metabolism as indicated by null mutations of MCH or MCH-R1 in transgenic mice, resulting in a lean phenotype and altered energy metabolism [19,20]. Our results indicate that MIZIP is co-expressed with MCH-R1 in most tissues, especially in brain, and is able to bind to the C-terminus of MCH-R1 in co-transfected cells. Therefore, it is tempting to speculate that MIZIP may be involved in MCH-R1 function, for example, as a regulatory molecule involved in spe-

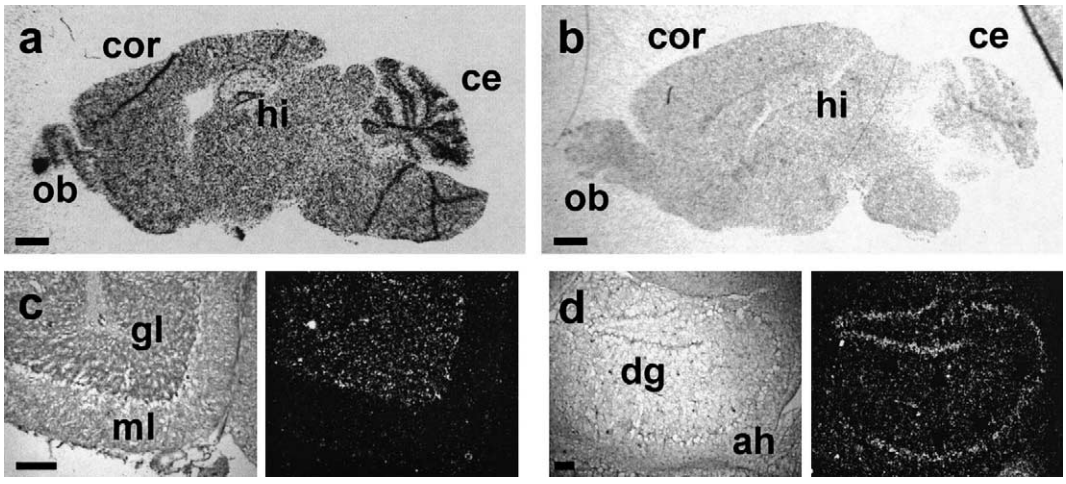

Fig. 5. RNA in situ hybridization analyses of MIZIP in adult mouse brain. a,b: serial sections of adult mouse brain hybridized with a α-[^{35}S]UTP-labeled MIZIP antisense (a) and sense (b) riboprobe and exposed to film are shown. Higher magnification of the cerebellum (c) and the hippocampus (d) of the brain shown in (a) as bright field (left) and corresponding dark field (right) images were obtained after dipping to photo emulsion, development and microscopic examination. ah, ammons horn; ce, cerebellum; cor, cortex; dg, dentate gyrus; hi, hippocampus; gl, granular layer; ml, molecular layer; ob, olfactory bulb. Bars: a,b, 1 mm; c–f, 100 μm.

cific signal transduction, desensitization or localization of MCH-R1.

Acknowledgements: Supported by the Deutsche Forschungsgemeinschaft (BA 2160/1-1) to D.B. and by the European Commission (QLG3-CT-1999-00908) to D.R.

References

[1] Beck, B. (2000) Nutrition 16, 916–923.
[2] Bächner, D., Kreienkamp, H., Weise, C., Buck, F. and Richter, D. (1999) FEBS Lett. 457, 522–524.
[3] Saito, Y., Nothacker, H. and Civelli, O. (2000) Trends Endocrinol. Metab. 11, 299–303.
[4] Hawes, B.E., Kil, E., Green, B., O'Neill, K., Fried, S. and Graziano, M.P. (2000) Endocrinology 141, 4524–4532.
[5] Brzostowski, J.A. and Kimmel, A.S. (2001) Trends Biochem. Sci. 26, 291–297.
[6] Milligan, G. and White, J.H. (2001) Trends Pharmacol. Sci. 22, 513–518.
[7] Lakaye, B., Minet, A., Zorzi, W. and Grisar, T. (1998) Biochim. Biophys. Acta 1401, 216–220.
[8] Schwärzler, A., Kreienkamp, H.J. and Richter, D. (2000) J. Biol. Chem. 275, 9557–9562.
[9] Hervieu, G.J., Cluderay, J.E., Harrison, D., Meakin, J., Maycox, P., Nasir, S. and Leslie, R.A. (2000) Eur. J. Neurosci. 12, 1194–1216.
[10] Roth, A., Kreienkamp, H.J., Meyerhof, W. and Richter, D. (1997) J. Biol. Chem. 272, 23769–23774.
[11] Zitzer, H., Hönck, H.H., Bächner, D., Richter, D. and Kreienkamp, H.J. (1999) J. Biol. Chem. 274, 32997–33001.
[12] Lerman, M.I. and Minna, J.D. (2000) Cancer Res. 60, 6116–6133.
[13] Lutterbach, B., Sun, D., Schuetz, J. and Hiebert, S.W. (1998) Mol. Cell. Biol. 18, 3604–3611.
[14] Sugihara, T.M., Bach, I., Kioussi, C., Rosenfeld, M.G. and Andersen, B. (1998) Proc. Natl. Acad. Sci. USA 95, 15418–15423.
[15] Gross, C.T. and McGinnis, W. (1996) EMBO J. 15, 1961–1970.
[16] Hervieu, G. and Nahon, J.-L. (1995) Neuroendocrinology 61, 348–364.
[17] Kokkotou, E.G., Tritos, N.A., Mastaitis, J.W., Sliker, L. and Maratos-Flier, E. (2001) Endocrinology 142, 680–686.
[18] Mori, M., Harada, M., Terao, Y., Sugo, T., Watanabe, T., Shimomura, Y., Abe, M., Shintani, Y., Onda, H., Nishimura, O. and Fujino, M. (2001) Biochem. Biophys. Res. Commun. 283, 1013–1018.
[19] Shimada, M., Tritos, N.A., Lowell, B.B., Flier, J.S. and Maratos-Flier, E. (1998) Nature 396, 670–674.
[20] Marsh, D.J., Weingarth, D.T., Novi, D.E., Chen, H.Y., Trumbauer, M.E., Chen, A.S., Guan, X.M., Jiang, M.M., Feng, Y., Camacho, R.E., Shen, Z., Frazier, E.G., Yu, H., Metzger, J.M., Kuca, S.J., Shearman, L.P., Gopal-Truter, S., MacNeil, D.J., Strack, A.M., MacIntyre, D.E., Van der Ploeg, L.H. and Qian, S. (2002) Proc. Natl. Acad. Sci. USA 99, 3240–3245.