FEBS Letters 480 (2000) 156–160 FEBS 24044

A δ opioid receptor lacking the third cytoplasmic loop is generated by atypical mRNA processing in human malignomas

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Received 9 June 2000; revised 1 August 2000; accepted 1 August 2000

Edited by Veli-Pekka Lehto

Abstract δ Opioid receptors were identified in human melanomas by RT-PCR and radioligand binding. In all tumors an additional PCR amplificate was detected in which 144 bp within the third exon were deleted. This fragment corresponded to the third cytoplasmic domain of the receptor protein. The short variant resulted from atypical mRNA processing. There were no common splice recognition sequences around the deleted fragment; instead its excision resembled the removal of a transposon. The deletion was not detected in normal human melanocytes nor in human or rat brain. However, it was present in a human neuroblastoma cell line (SH-SY5Y). Thus, it appears that the occurrence of the short δ opioid receptor is correlated to malignancy.

Key words: δ Opioid receptor; Melanocyte; Melanoma; Skin; Neuroblastoma; mRNA processing

1. Introduction

δ Opioid receptors, along with μ and κ, belong to the family of neuropeptide receptors which respond to endogenous opioid peptides [1]. They are mainly found in the central and peripheral nervous system. However, in the dermis of frogs opioid peptides with a high selectivity for δ or μ receptors were detected and called deltorphins and dermorphins, respectively [2,3]. There is evidence for a local opioid system in human skin, too. The presence of opioid binding sites has been reported [4] as well as effects of opioids on human keratinocytes [5]. Furthermore, the opioid peptide β-endorphin was found in cultured human epidermal cells [6,7] as well as the mRNA for its precursor proopiomelanocortin (POMC) [8]. Correspondingly, binding sites for β-endorphin became evident on human keratinocytes [9]. The epidermal part of the human skin mainly consists of keratinocytes. A small fraction is made up of melanocytes which are sending out dendrites around which a group of several keratinocytes is located to receive melanophores from the former [10]. In this work the presence of δ opioid receptors in normal human melanocytes will be demonstrated.

The rat δ opioid receptor was cloned in 1992 [11,12], and the human form was published 2 years later along with its genomic organization [13]. The coding region consists of three exons separated by two introns, 46.3 kb and 3.4 kb long. Each of these introns is flanked by the usual splice donor and acceptor sequences, i.e. GT followed by purines at the 5′ ends and AG preceded by a pyrimidine-rich stretch at the 3′ ends [14–16]. In the present study it will be shown that in certain tumor cells an atypical mRNA processing takes place which does not involve such motifs.

2. Materials and methods

2.1. Reagents

Cell culture reagents were from Gibco, and enzymes for molecular biology including reaction buffers were from Hybaid-AGS (Heidelberg, Germany) unless stated otherwise. The opioid receptor ligands DPDPE (cyclic [D-penicillamine^2,D-penicillinamine^5]) enkephalin) and DAMGO ([D-Ala^2,MePhe^4,Gly-ol^5] enkephalin) were from Bachem, Heidelberg, Germany, and U 69 593 ((5α,7α,8β)-(+)-*N*-methyl-*N*-(7-[1-pyrrolidinyl]-1-oxaspiro[4,5]dec-8-yl)-benzeneacetamide) was from Sigma, Deisenhofen, Germany. The corresponding radiolabeled ligands were from NEN Life Science Products, Boston, MA, USA.

2.2. Cell culture

Primary human normal melanocytes prepared as described previously [17,18] were a generous gift from Dr. S. Kippenberger, Frankfurt/Main, Germany. The cells were received at the 11th passage and kept for further passages in our laboratory in Ham's F10 medium supplemented with 5% fetal calf serum (FCS), 85 nM TPA (4-*O*-methyl-12-*O*-tetradecanoylphorbol-13-acetate), 0.1 mM isobutylmethylxanthine (IBMX) and 0.1 nM cholera toxin in a water-saturated atmosphere at 37°C and 5% CO_2. G361 cells (ECACC, Salisbury, UK) were cultured in McCoy's 5A medium containing 10% FCS, the lines COLO 679 (ECACC, Salisbury, UK) and COLO 800 (DSMZ, Braunschweig, Germany) were kept in RPMI 1640 medium supplemented with 10% FCS under the same atmospheric and temperature conditions as the melanocytes. SH-SY5Y neuroblastoma cells (DSMZ, Braunschweig, Germany) were grown in DMEM medium containing 15% FCS.

2.3. Radioligand binding assay

2.3.1. Membrane preparation. Crude membrane fractions were prepared following described protocols [19,20] with minor modifications. All steps were carried out at 0–4°C. G361 cells grown to confluency on petri dishes were washed twice with homogenization buffer (10 mM Tris–HCl, pH 7.4, 0.3 M sucrose) and scraped off in this buffer containing a protease inhibitor mix (0.01% aprotinin, 1 mM PMSF, 10 μg/ml leupeptin, 50 μg/ml bacitracin, 10 μg/ml soybean trypsin inhibitor). Homogenization was performed in a glass–teflon homogenizer by 20 strokes. The homogenate was centrifuged at $200\times g$ for 5 min to remove nuclei and cell debris; the supernatant was centrifuged twice at $38\,000\times g$ for 20 min. The resulting pellet represented the crude membrane preparation. It was processed immediately or was stored at −80°C until use.

2.3.2. Binding assay. G361 cell membranes were incubated in binding buffer (0.5 mM $MgCl_2$, 2.7 mM KCl, 140 mM NaCl,

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PII: S0014-5793(00)01929-3

1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4), containing 0,1% BSA and the protease inhibitor mix described above, together with one of the labeled ligands, [^{3}H]DPDPE, [^{3}H]DAMGO or [^{3}H]U69593 in concentrations of 2.2, 1.8 and 2.5 nM, respectively. To determine non-specific binding to the membranes, part of the samples also contained the corresponding unlabeled ligand in a concentration of 20 μM unless stated otherwise. Samples (100 μl) were incubated for 120 min on ice; then free radioactivity was removed by sample filtration through GFB glass fiber filters (Whatman-Biometra, Göttingen, Germany) which had been pre-soaked in 0,1% polyethylenimine at room temperature for 3 h. Filters were washed three times with 3 ml ice-cold binding buffer containing 0.01% Triton X-100. The filters were transferred to scintillation vials, and radioactivity was determined by liquid scintillation counting. Samples were run in triplicate; membranes from 3×10^7 cells were used per sample.

2.4. RNA extraction and PCR amplification

Total RNA was extracted obeying standard protocols using guanidinium thiocyanate and sodium lauryl sulfate in citrate buffer for lysing the cells or tissue followed by phenol–chloroform treatment and ethanol precipitation. This RNA preparation also served as a template for genomic PCR because it contained sufficient DNA. To prevent amplification of the latter in the RT-PCR setups, all PCR primer pairs intended for amplifying cDNA spanned at least one intron which excluded genomic amplification. Complementary DNA was reverse-transcribed from total cellular RNA by using random hexanucleotide primers. Reactions were performed for 15 min at 18°C and then for 2 h at 42°C. The total reaction volume was 20 μl which contained 90 U of enzyme and 1–3 μg of RNA. Four microliters of this reaction mixture were used as a template for PCR amplification. The total PCR reaction volume was 20 μl. The reaction mixture consisted of PCR buffer (Appligene, Heidelberg, Germany, for *Taq* polymerase or Promega, Mannheim, Germany, for *Pfu* polymerase), 0.2 mM of each dNTP, 4 pmol of each primer and 0.8 units of *Taq* or *Pfu* (Promega, Mannheim, Germany) DNA polymerase. The following primers were used, an overview is also given in Fig. 1.

Primer 1: 5′-CAA GGC CCT GGA CTT CCG CA, position 462; primer 2: 5′-TCC CAA GCC TCC GAC CTT CTA CTG, position 1234, complementary strand. PCR conditions for this primer pair: 2 mM $MgCl_2$, annealing temperature 66°C.

Primer 3: 5′-GCC AAG CTG ATC AAC ATC TG, position 493; primer 4: 5′-AAG CAG CGC TTG AAG TTC TC, position 986, complementary strand; primer 5: 5′-ACC AAG ATC TGC GTG TTC CT, position 637. PCR conditions for the primer combination 3/4: 1.5 mM $MgCl_2$, annealing temperature 58°C; conditions for primer combination 5/4: 2 mM $MgCl_2$, annealing temperature 58°C.

Primer 3a: 5′-GTT GGC GTG CCC ATC ATG GT, position 535; primer 4a: 5′-GAA GTT CTC GTC GAG GAA AGC GTA G, position 975, complementary strand. This primer pair was used for nested reamplification of primers 3/4 amplificates. PCR conditions for 3a/4a: 2 mM $MgCl_2$, annealing temperature 68°C.

Primer 7: 5′-GGG CTG CTG GGC AAC GTG CT, position 187; primer 8: 5′-CCA GAC GAT GAC GAA GAT GTG GAT GG, position 852, complementary strand; primer 9: 5′-AAG CAG CCG GTG GAG AGG GAC, position −40. PCR conditions for primers 7/8: 1.5 mM $MgCl_2$, annealing temperature 72°C; conditions for primers 9/8: 3 mM $MgCl_2$, annealing temperature 72°C.

Further primers used for genomic amplification flanked the third exon [21], or the left (5′) primer was placed in the intron upstream of exon 3. All PCR reactions started with a denaturation step of 3 min at 95°C followed by 40 cycles, 50 s 95°C, 90 s annealing (at the temperatures stated above) and elongation at 72°C for 3–6 min, depending on the length of the PCR product.

3. Results

3.1. δ Opioid receptors in pigment cells

Three human melanoma cell lines, G361, COLO 679 and COLO 800, were screened by RT-PCR for the presence of δ opioid receptor mRNA. The primers used, called primer 1 and 2, spanned about two thirds of the coding region including the stop codon (Fig. 1 gives a schematic representation of the δ receptor mRNA and the position of the primers used). From all three cell lines a PCR product of the expected length, 773 bp, could be created (Fig. 2A). Remarkably, an additional band, approximately 150 bp shorter, was present in all cases (see Section 3.2).

To confirm that the δ receptor mRNA from these pigment cells contained the complete coding region, three further primers were employed. They were called primer 7, 8 and 9, respectively, and were combined to two pairs, 7/8 and 9/8 (Fig. 1). The resulting amplificates encompassed the 5′-part of the coding region and the site of the deletion. In either case an amplification product of the correct length was obtained along with a band approximately 150 bp smaller. This finding indicates that pigment cells express the complete message for the δ opioid receptor and that the short form does not display further deletions. To exclude the possibility that the short fragment originated from a reading error of *Taq* polymerase, PCR reactions were also performed with *Pfu* polymerase which possesses proof-reading activity and therefore a lower

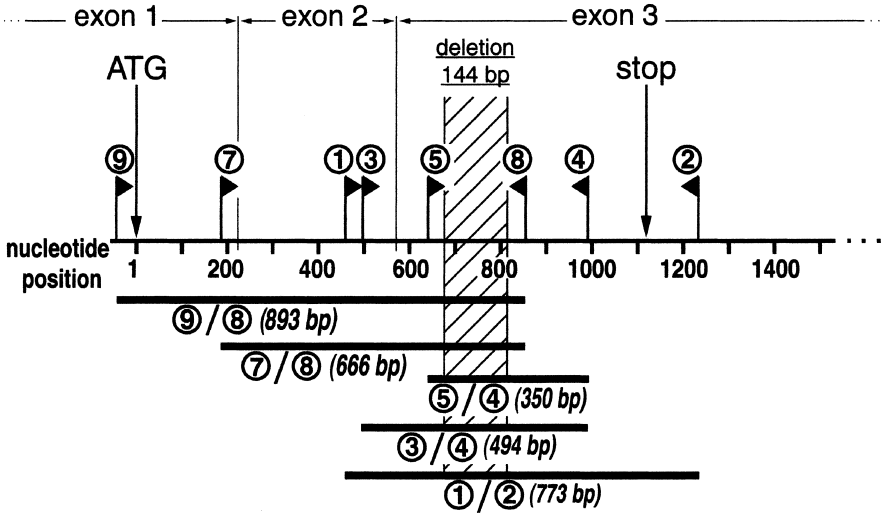

Fig. 1. Schematic representation of the δ opioid receptor cDNA along with the positions of the PCR primers used. PCR primers are shown as arrowheads. Primer pair 3a/4a used for nested reamplification is not indicated; it is located close to primers 3 and 4. Below the horizontal axis the length and position of the three amplificates created by the primers displayed are shown to scale.

error rate. An example for an amplification of cDNA from G361 cells using *Pfu* polymerase and primer pair 7/8 is given in Fig. 2B. Again, both bands were observed.

The presence of functional opioid receptors on pigment cells was demonstrated by ligand binding studies. They were performed with membranes of G361 cells, and tritium-labeled opioid agonists selective for μ, δ or κ receptors were employed (see Section 2 for details). Specific and reversible binding was detected with the δ receptor ligand DPDPE. Radioligand competition studies with increasing concentrations of unlabeled DPDPE (Fig. 2C) revealed a dissociation constant K_d of 6.2 nM (mean of three experiments). This value is in good accordance with reported K_d values for DPDPE on δ opioid receptors [11,12,22]. The receptor density was in the range of 10 fmol/mg protein, which is lower than the values reported for e.g. SH-SY5Y cells (around 35 fmol/mg protein according to [23] and in the range of 100 fmol/mg protein according to [24]). No specific binding was detected with the μ receptor specific ligand DAMGO nor with the κ ligand U 69 593 (not shown).

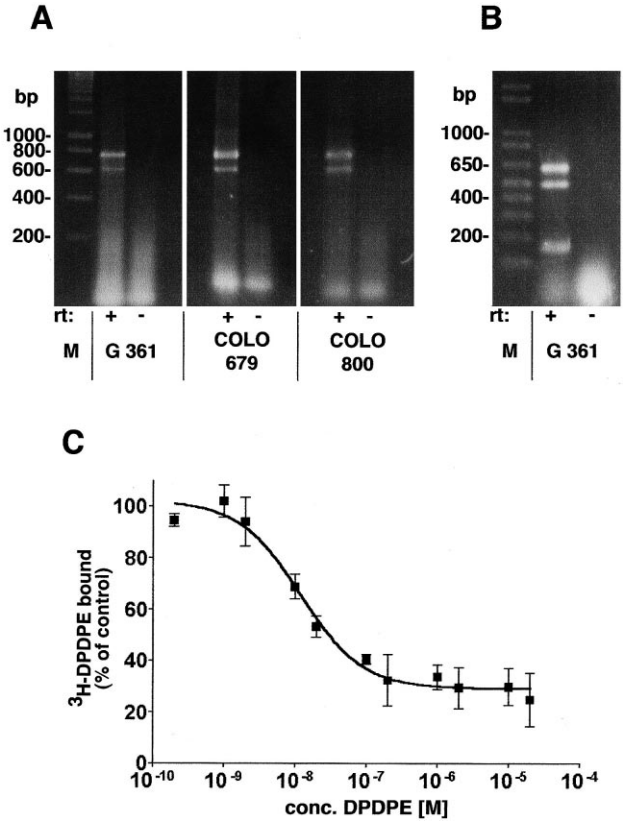

Fig. 2. Detection of δ opioid receptors in pigment cells by RT-PCR and by ligand binding. A: RT-PCR: primer pair 1/2 was used. As a control for contamination, reverse transcription reaction mixtures were set up omitting the enzyme (indicated as '−rt'). The cells lines tested are indicated. 'M' denotes the size marker lane. B: RT-PCR using proof-reading *Pfu* polymerase: to exclude reading errors by *Taq* polymerase as the cause of the smaller δ receptor band, *Pfu* instead of *Taq* polymerase was used to amplify a δ opioid receptor sequence from G361 cDNA (primer pair 7/8). C: Ligand binding: competition curve, displaying the inhibition of [^{3}H]DPDPE binding to G361 cell membranes by 10^{-10}–10^{-4} M concentrations of unlabeled DPDPE. [^{3}H]DPDPE binding is given in percent of control, i.e. [^{3}H]DPDPE binding in the absence of cold ligand. Each data point represents the mean ± S.E.M. of three independent experiments.

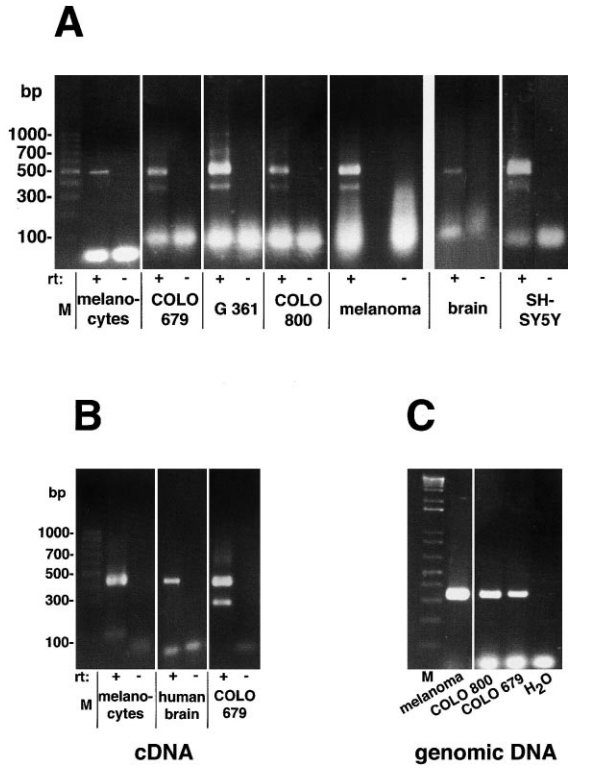

Fig. 3. Occurrence and origin of the short δ opioid receptor variant. A: Expression in different cell types: the primer pair 3/4 was used to screen the cell lines and tissues as indicated. The band at around 500 bp corresponds to the 'wild type' receptor. Correspondingly, the shorter form migrated at around 350 bp. 'Melanocytes' means primary cultured normal human melanocytes; COLO 679, G361, COLO 800 are three different human melanoma cell lines, and 'melanoma' indicates a native human melanoma metastasis obtained by surgery; 'brain' stands for rat frontal cortex. SH-SY5Y is a human neuroblastoma cell line. The omission of reverse transcriptase ('−rt') served as a control to rule out PCR contamination. 'M' denotes the size marker. B: Reamplification of selected samples from A, the nested primers 3a and 4a were used for reamplification to avoid overlooking small amounts of the short δ receptor in non-malignant tissues (melanocytes and human temporal cerebral cortex). For comparison, the result for the melanoma line COLO 679 is also shown. C: Genomic PCR amplification: the primers 4 and 5 were used which produce a 350 bp amplificate from the wild type sequence. 'M' denotes size marker, 'melanoma' is the same melanoma tissue as in A. Water as template served to exclude PCR contamination. Note that no additional band is visible indicating that the genome contains no sequence corresponding to the shorter form of the δ receptor.

3.2. *Distribution of the short form of δ opioid receptors in pigment and neuronal cells*

Further cells and tissues were screened for the expression of the novel short form of the δ opioid receptor, namely normal human melanocytes [17,18], rat and human brain, a native human melanoma metastasis obtained from a Caucasian patient and a human neuroblastoma cell line (SH-SY5Y). Fig. 3A gives an overview of the results. The normal δ opioid receptor, identified by a 500 bp amplificate (primer pair 3/4), was present in all cases. The short form (350 bp amplificate), however, was exclusively detected in melanoma and neuroblastoma cells, i.e. in all malignant tumors tested. On the other hand, neither normal melanocytes nor normal brain did express the short variant. Several areas of the rat cerebrum were tested (frontal cortex is shown in Fig. 3A) as well

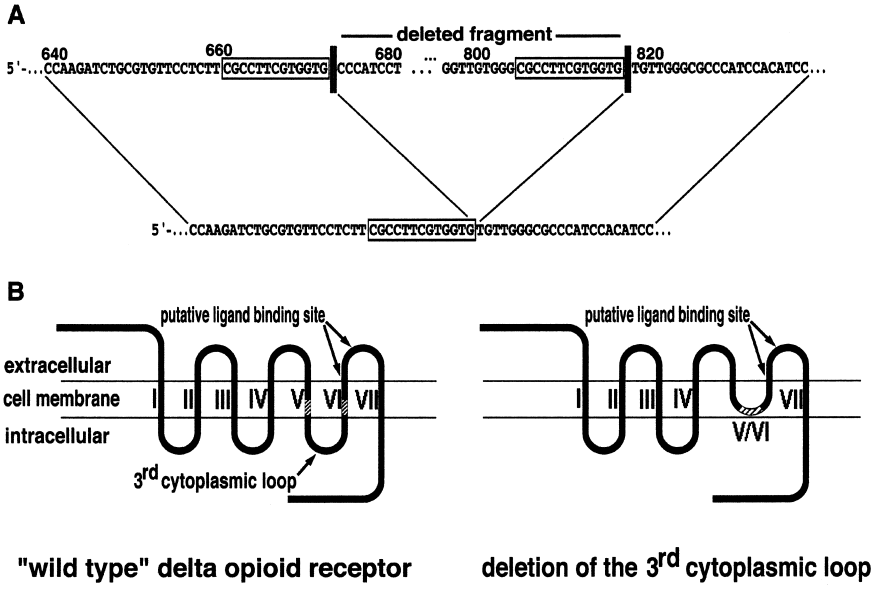

Fig. 4. Position of the 144 bp deletion in δ opioid receptor cDNA and its putative implications for the receptor molecule structure. A: Shows the human δ opioid receptor cDNA sequence surrounding the deletion site (nucleotide position 673–816). Upper line: 'wild type' sequence; the fragment to be deleted is indicated. The lower line shows the resulting cDNA sequence after removal of the 144 bp stretch. Boxed are the 13 bp motifs that are flanking the deletion site on both sides. B: The molecular structure of the δ opioid receptor protein is depicted schematically. The small hatched boxes within the peptide chain indicate the positions that correspond to the 13 bp motifs boxed in part A. Left: wild type receptor, right: short form.

as human temporal cortex (Fig. 3B), and in no case the short δ receptor mRNA was detected. To exclude that the latter was overlooked in non-malignant tissue because of low abundance, we performed reamplification reactions with nested primers (primers 3a and 4a) using PCR products obtained with the primers 3/4 as templates (Fig. 3B). This experiment clearly confirmed the absence of abnormal δ receptor mRNA in non-malignant cells.

3.3. Origin of the short δ opioid receptor variant

In order to investigate how the short δ opioid receptor is produced, genomic DNA was amplified in search for aberrations within the δ receptor gene (Fig. 3C). Only a single PCR product was obtained for each sample, and this product had the length that was expected for the normal receptor (350 bp). Also, it revealed no sequence abnormalities. This means that the short δ receptor form is not encoded in the genome; consequently, it must have been produced by mRNA processing. Nucleotide sequencing of the short receptor cDNA revealed a deletion of 144 nucleotides (bases 673–816, Fig. 4A) within the third exon. The length and position of the deletion were identical in all samples tested. No other mutations were detected. No frame shift resulted from this deletion. Therefore, the receptor protein can be predicted to carry a deletion of 48 amino acids.

The δ opioid receptor belongs to the family of G-protein coupled receptors which possess seven transmembrane domains. It can be derived from published data [13] that the missing 48 amino acids exactly correspond to the third cytoplasmic domain of the receptor molecule (Fig. 4B). Remarkably, no splice consensus motifs were found at the respective ends of the deleted fragment. Instead, another feature became evident at the boundaries of the deletion. The region to be excised was flanked by two copies of a 13 bp nucleotide stretch (CGCCTTCGTGGTG, boxed in Fig. 4A). One of these copies became deleted during the removal of the 144 bp fragment whereas the other remained in place (Fig. 4A). Insertion of a nucleotide sequence by doubling several bases at the insertion site and then removing the sequence again by undoing the duplication is the mechanism by which all known transposable DNA elements (transposons) enter a site of genomic DNA and leave it again [25]. Thus, a similar mechanism seems to be at work in the case of the δ opioid receptor.

4. Discussion

The results described above demonstrated the existence of a to date unidentified variant of the δ opioid receptor which is generated by atypical mRNA processing, and the occurrence of which is correlated with malignancy. The variant lacked 144 nucleotides, which corresponded to the third cytoplasmic loop of the receptor protein, and was found exclusively in malignant cells. The position and length of the deletion was the same in all tumors tested, and other variations were not found. These observations make it unlikely that the excision of 144 bp is due to undirected mRNA processing in malignant cells. Other variants of δ and κ opioid receptors were reported recently [26]. In this case, however, the receptor proteins were truncated, and the cause was clearly alternative splicing. To date, no information about the physiological or pathophysiological role of these receptor fragments is available.

The structure–function relationships of G-protein coupled receptors, to which family the δ opioid receptor belongs, are quite well understood. It its known for G-protein coupled receptors in general that the third cytoplasmic loop is involved in G-protein coupling and in receptor desensitization after chronic agonist stimulation [27]. Studies dealing with the δ opioid receptor revealed a potential ligand binding site in the third extracellular loop extending into the sixth transmembrane domain [22,28,29], depicted schematically in Fig. 4B.

The ligand binding site is localized immediately adjacent to the third cytoplasmic loop. Therefore, signal–response coupling could be affected. Alternatively, the truncated δ receptor may act by heterodimerization or hetero-oligomerization with the wild type form and thereby disturb the function of the latter as recently described for mutated D2 dopamine receptors [30]. Melanocytes could be exceptionally sensitive to any disturbance of intracellular signaling since keeping them in culture requires the permanent activation of intracellular signaling pathways by TPA, IBMX and cholera toxin added to the culture medium [31,32]. It is known that mutated signaling molecules (e.g. $G_s\alpha$) are able to induce tumor growth [33,34]. Thus, further studies will reveal whether the short δ receptor is involved in tumor genesis.

Acknowledgements: This work was supported by the Land Sachsen-Anhalt. We thank Dr. S. Kippenberger for providing primary cultured human melanocytes and Drs. T. Feuerstein and T. Freiman for providing human brain tissue.

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