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Single amino acid variation underlies species-specific sensitivity to amphibian skin-derived opioid-like peptides

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Abstract

It has been suggested that the evolution of vertebrate opioid receptors (ORs) follow a vector of increased functionality. Here we test this idea comparing human and frog ORs. Interestingly, some of the most potent opioid peptides known have been isolated from amphibian skin secretions. Here we show that such peptides (dermorphin and deltorphin) are highly potent in the human receptors and inactive in frog ORs. The molecular basis for the insensitivity of the frog ORs to these peptides was studied using chimeras and molecular modeling. Interestingly, the insensitivity of the delta opioid receptor (DOR) to deltorphin was due to variation of a single amino acid– Trp7.35—which is a leucine in mammalian DORs. Notably, Trp7.35 is completely conserved in all known DOR sequences from lamprey, fish and amphibians. The deltorphin-insensitive phenotype was verified in fish. Our results provide a molecular explanation for the species selectivity of skin-derived opioid peptides.

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INTRODUCTION

Opioid receptors (ORs) mediate the analgesic and antinociceptive effects of endogenous opioid peptides and exogenous opioid small molecules in vertebrates [1–3]. The three classic opioid receptors, designated μ, δ and κ (MOR, DOR and KOR), were originally characterized by the pharmacological profiles of their responses to both shared and type-specific ligands [1]. The genes for these three ORs, along with the related nociceptin receptor, occur on separate chromosomes in most known vertebrate genomes [1, 2]. Sequence-based studies of ORs have suggested that these four ORs arose via two genome-wide pre-Mesozoic duplication events [2, 4–7].

Early studies of the analgesic and antinociceptive effects of opioid compounds in amphibians and fish provided evidence for the existence of opioid-like receptors in these organisms [3, 8–12], although these receptors differed pharmacologically from their mammalian orthologs. One of the first lines of evidence for this was derived from studies of KOR-like sites in the brain of the edible frog (*Rana esculenta*), in which it was reported that the KOR-like binding sites had higher affinities for MOR- and DOR-specific compounds than mammalian KOR receptors [13, 14]. Intriguingly, the degree of stereo-selectivity for arylacetamide and benzomorphan-derived ligands was also less at frog KOR than at the mammalian receptors [15].

All four opioid receptors have subsequently been cloned from additional amphibian and fish species [6, 16–23], and genome sequencing projects have revealed these same receptors in a large number of other species. The sequences of the four types of opioid receptors are highly conserved among species, with the most striking differences between the mammalian and non-mammalian receptors being in the extracellular loop domains [2, 6]—regions considered to constitute the “selectivity filter” of opioid receptors [24, 25]. In general, pharmacological studies have revealed that opioid receptors from mammals are more selective than those from non-mammals, [21–23, 26] leading to the hypothesis that opioid receptor type-selectivity exhibits an evolutionary vector of increased selectivity from fish and amphibians to mammals [2]. Among the many striking differences between the opioid systems of mammals and amphibians is the presence of highly potent endogenous opioid peptides (deltorphins [27] and dermorphins [28]) in the skin of many amphibians and mollusks [29]. Here we show that, although opioid-like peptides from amphibian epidermal secretions are remarkably potent at the human receptors, they do not activate the frog receptors. Further, by using human–frog chimeric receptors, site-directed mutagenesis and molecular modeling of newly-solved structures of opioid receptor-peptide complexes, we established the molecular basis of these differences. Our results predicted that DORs from Lamprey, Fish and Amphibian (LFA) sources would be insensitive to deltorphin, and we confirmed this prediction by showing that they are inactive in zebrafish *in vivo*.

Results

Pharmacological comparison of human and frog (Rana pipiens) opioid receptors

In preliminary experiments, we tested the expression levels of three human (hKOR, hMOR, hDOR) and three frog (*Rana pipiens;* rpKOR, rpMOR, rpDOR) opioid receptors via

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saturation binding assays using ^{3}H-diprenorphine (see Methods for details). All transfected receptors displayed high affinity ^{3}H-diprenorphine binding (K_D's ranged from 0.6 to 2.2 nM), with high expression levels (βmax ranged from 2–8 pmol/mg) (Table 1), facilitating the comparison of functional data between species.

We used a previously described $G_{\alpha i}$ assay [30–34]to characterize the differential selectivity profiles of *Rana pipiens* versus human ORs (Table 2). For these studies, we evaluated the agonist potencies and efficacies of 14 agonists at each of the three different ORs from *Rana pipiens* and humans. In most cases when comparing human and frog ORs, agonists maintained their type selectivity albeit with lower potencies at the frog receptors. Thus, for example, the δ-selective ligand DADLE ([D-Ala^2,D-Leu^5]-enkephalin) was 90-fold less potent at the frog than at the human DOR. Similarly the μ-selective agonist DAMGO ([D-Ala^2,*N*-MePhe^4,Gly-ol]-enkephalin) was 300-fold less potent at the frog than at the human MOR. Interestingly, the differences between frog and human receptors were less pronounced when naturally occurring mammalian opioid peptides were evaluated. Thus, for example, the endomorphins were 20-fold less potent at the frog MOR than at the human MOR. Interestingly, whereas all of the tested KOR-specific peptides (dynorphin A, dynorphin B and α-neoendorphin) were 10-fold less potent at the frog KOR, the amphibian dynorphin xendorphin [35, 36] was equally active at frog and human KORs. Type selectivity was mostly maintained in frog relative to human ORs. However, in the case of the synthetic peptides DADLE and DAMGO, type selectivity differed between species (see Table 2 for details). By contrast, the KOR-selective dynorphins, as well as the small molecule salvinorin A, were similarly selective in human and amphibian KORs.

The most pronounced differences between the human and frog receptors were seen with the dermorphin and deltorphin peptides, which are secreted in the skin of the tree frog-*Phyllomedusa bicolor.* As previously documented, dermorphin is a potent and selective human MOR agonist, and deltorphin II and deltorphin C are potent and selective human DOR agonists. Deltorphin II and deltorphin C were inactive at the three tested frog ORs (Fig 2B; Table 2) while dermorphin was an exceedingly weak agonist (Fig 2A; Table 2).

Identification of the molecular determinants of the pharmacological differences between frog and human opioid receptors

We next sought to determine the molecular basis for this striking species selectivity. An analysis of the sequences of human and frog receptors revealed that, for the most part, the major differences between these ORs reside in the extracellular loops and the receptor termini [6] (Fig. 1, Supp. Fig. 1). We hypothesized that the functional differences between human and frog ORs stem from differences in their corresponding sequences. Therefore, to characterize the molecular basis for the pharmacological differences between the frog and human MORs and DORs, a series of chimeric receptors was made to produce frog ORs with human "inserts" in various transmembrane and extracellular domains (Fig. 1B and 1C; Supplementary Table 2), covering most of the differences between the species, except for the N- and C- termini. The chimeras were designed to explore the idea that sequences in certain regions of the human receptors may be critical for their increased sensitivity

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compared to their frog homologs. The new chimeras and mutants explored most of the differences between human and frog ORs, except for the N- and C- termini.

(a) Mu opioid receptors—Four chimeras (Fig. 1B–1 to 4) were made that swapped single stretches of human MOR sequences into the frog MOR. Functional analyses of the responses of these four chimeras to endomorphin, dermorphin or DAMGO showed that their responses were essentially identical to the wild-type frog MOR. Since the N-terminus of the human MOR had been implicated in the binding of opioid peptides [37], we constructed 3 additional MOR chimeras (Fig. 1B-5 to 7) by "humanizing" the N termini of three constructs (residues 1 to 220 of WT rpMOR, rpMOR EL2h and rpMOR EL3h). These chimeras, h-rpMOR-WT, h-rpMOR-EL2h and h-rpMOR-EL3h were more responsive to the tested peptides than the WT rpMOR (Fig. 2A and Table 3). Moreover, the "humanization" of different regions had differential effects on the responses to various peptides – the activity of DAMGO, endomorphin 1 and 2 in rpMOR was partially rescued by humanization of the N-terminus, and even further enhanced by humanization of EL3h, but not by EL2h (Table 3). Interestingly, the activity of dermorphin with the chimeric MOR followed a different pattern (Table 3 and Fig. 2A); humanization of the N-terminus rescued receptor activation by dermorphin, which was slightly enhanced by humanization of EL3 (~2-fold) and markedly enhanced by humanization of EL2 (~10-fold). We also examined the affinity of DAMGO and dermorphin to these chimeras using a competition binding assay with radiolabeled diprenorphine (Fig. 2A and Table 3). Interestingly, the affinity of DAMGO was not altered between the frog MOR and the three chimeras (K_i range 100–200 nM), whereas the affinity of dermorphin to rpMOR was increased by ~30-fold only in the h-rpMOR EL2h chimera, an effect that correlated well with the functional data.

(b) Delta opioid receptors—Five DOR chimeras (Fig. 1C-1 to 5), in which single stretches of the frog DOR were "humanized", were constructed and tested by functional and binding studies with peptide ligands (Table S3 and Fig. 2B). Changing TM4 or TM5 from the frog sequence to the human sequence (Fig 1C–1 and 3) slightly reduced the activity of all four tested peptides, despite the fact that the human wild type receptor is more responsive to these ligands than the frog wild type receptor. Changing TM6 from frog to human (Fig. 1C–4) slightly increased the potencies of all four peptides (Table S3). Changing EL2 from frog to human increased the potencies of DADLE, and the deltorphins, but not enkephalin (Table S3, Fig. 2B). Changing EL3 from frog to human increased the potencies and the affinities of all of the tested peptides.

To further explore the role of the EL3 region in DOR activity, we mutated several residues of interest in EL3 and the beginning of TM7 of rpDOR to their corresponding human residues. The mutants Met287Leu, Asn294Asp and Tyr296Leu maintained the rpDOR phenotype, *i.e.*, they did not respond to deltorphin (data not shown), but the mutant Trp301Leu (7.35 in the numbering scheme of Ballesteros and Weinstein [38]) responded to deltorphin with substantially increased potency and affinity compared to rpDOR; this mutation also partially rescued activation by the other tested peptides (Table S3).

Structural basis of W 7.35 role in deltorphin selectivity—To evaluate the importance of position 7.35 in opioid receptors, we tested the effect of the reverse mutation

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in the human DOR (Leu300Trp). Significantly, the L300W mutation diminished deltorphin potency nearly 2000-fold (Fig. 4). Bioinformatic analysis of DORs from multiple species reveals an interesting pattern – all of the known sequences of DOR from LFA (Lamprey, Fish and Amphibians) contain tryptophan at position 7.35 (Fig. 3A and B).

To elucidate a structural explanation for the role of W7.35 we next performed docking studies of deltorphins into the wild-type human DOR in complex with DIPP-NH2 tetrapeptide (PDB – 4RWD) [39]and to a modeled L3.75W mutation. In this study, DIPP-NH2 peptide has been found to have weak partial agonist activity at human (but not murine) DOR, though this entails only minor changes in the receptor binding pocket as compared antagonist naltrindole bound DOR. Therefore, the structure of DOR bound to a DIPP-NH2 can provide information about the initial interaction between peptide agonists and the receptor. Both deltorphin C and deltorphin II are predicted to bind to the human DOR in similar binding poses, characterized by several key interactions with the receptor (Figure 3D). Notably, the N-terminal amino group forms a salt bridge with the Asp128(3.32) side chain carboxylate group, while the tyrosine aromatic ring occupies a hydrophobic core pocket, with its hydroxyl group making polar interactions with ordered waters found in high resolution DOR structures. We note that inclusion of these water molecules in the structural model was important for consistently high-scoring docking of the peptide. Residues 1 to 4 of the ligand backbone adopt a well-defined turn conformation, which positions the D-Ala2 and Phe3 side chains in hydrophobic subpockets, and allows the acidic side chain of Asp/Glu4 to extend back into the pocket to form additional interactions with the N-terminal amino group and the Tyr56 side chain. Interestingly, free dynorphin peptides were predicted to preferentially adopt a very similar conformation for residues 1 to 4 in extensive energy optimizations. The C-terminal residues 5 to 7 are less defined in our docking, with several possible low energy conformations predicted for each peptide. Importantly, residue 7.35 is positioned at the entrance to the binding cavity and Leu7.35 in the human DOR comprises a part of the hydrophobic pocket that accommodates the benzene ring of the peptide Phe3 [39]. In the frog DOR, however, mutation to a more bulky Trp7.35 side chain results in a prominent steric clash between its indole ring and Phe3. Attempts to dock deltorphins to the hDOR(Leu7.35Trp) mutant and a frog DOR model resulted in less consistent ligand poses and degraded binding scores. Taken together, these modeling results support a prominent role for residue 7.35 as a “selectivity filter” in DOR.

Zebrafish are insensitive to deltorphin—The mutagenesis and bioinformatic results suggested that the insensitivity of rpDOR to deltorphin was likely to be true of DORs from most, if not all, LFA. To test this prediction, an independent model system in which deltorphin affinity and potency had never been tested was selected; to that end, we tested the effect of DOR agonists on zebrafish swimming behavior. We treated larvae, 7 days post fertilization (dpf), with deltorphin or enkephalin peptides for 1 hour and quantified their swimming behavior (Fig. 5). When zebrafish were treated with either of the enkephalin peptides, we observed dose-dependent decreases in swimming levels. In contrast, the deltorphins had no significant effect on zebrafish swimming. We conclude that zebrafish are specifically sensitive to the enkephalin peptides and not deltorphins, likely due to the presence of W rather than L at position 7.35 of the zebrafish DOR.

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Discussion

The main finding of this paper is that frog-derived opioid peptides show remarkable species-selectivity which is specified –in the case of DOR—by a single amino acid change which has been conserved in all higher vertebrates. Clearly a functional comparison of orthologous receptors from different species can facilitate our understanding of the relationship between structure and function of these receptors; in addition, such comparisons may help us to understand how these receptors evolved, and the pressures that may have been exerted to promote such evolution. In the current study, we have verified that all of the frog and human receptors were functionally expressed in HEK293-T cells, and were coupled to $G\alpha_i$, as would be predicted on the basis of sequence identity in the intracellular loop regions of opioid receptors from frogs and humans. We showed that, in most cases, human opioid receptors respond to both naturally occurring and synthetic peptides with higher potencies than opioid receptors from *Rana pipiens*. This is true in all of the examined cases except for the *Xenopus laevis*-derived peptide xendorphin, which demonstrated a comparable KOR-specific activity in both human and frog KOR, and very low to no activity at MOR and DOR receptors. The KOR specificity of xendorphin has been previously demonstrated by displacement of a KOR radioligand from rat whole brain membranes [36].

The lower potencies observed for all of the frog ORs (Table 2) are consistent with previous reports characterizing other cloned non-mammalian ORs, including those from white suckerfish [16] and rough-skinned newt [21]. But in the case of the *Xenopus* dynorphin, xendorphin, we observed that, although frog receptors are not highly activated by mammalian peptides, the human receptors may be “compatible” with the amphibian peptides. Together, these data support the concept of an ‘evolutionary vector’ that leads from receptors with low responsiveness (low potency) in non-mammalian aquatic or amphibian vertebrates to those with high responsiveness and selectivity in mammals.

One of the initial motivations for this work was the observation that certain amphibians can secrete large quantities of extremely potent psychoactive compounds and peptides from their skin without apparent ill effects. It has been proposed that such secretions serve to protect against predators in nature [40, 41] and in other cases, it has been shown that the evolution of such natural defensive and survival strategies is often accompanied by resistance or insensitivity to certain natural materials [42–46]. As we show here, the amphibian opioid receptors we tested are insensitive to deltorphin and dermorphin and, in the case of deltorphin, this is due mainly to a difference in sequence at a single conserved residue—leucine (mammalian) vs tryptophan (LFA) at position 7.35. Recent structural studies of antagonist-bound human MOR and DOR [30, 47, 48] show that residue 7.35 (Trp318 and Leu300, respectively) lies at the entrance to the binding site and is likely to be a part of the receptor specificity determinant [30, 47, 49, 50]. The Leu7.35Trp mutation in human DOR resulted in decreased affinity for some of the DOR-specific ligands [51], whereas mutations of the conserved tryptophan in MOR suggested the key role of this residue for MOR specificity [52, 53]. As opioid-like peptides from frog skin are thought to be a natural defense mechanism [40, 41], it would thus be advantageous for frogs that these peptides are inactive, although one would expect them to activate the receptors of potential frog predators.

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A comparison of the structures of human DOR and MOR [30, 47, 49, 50] revealed that there are 14 residues within 4 Å of the bound ligand, 11 of which are identical between the two receptors; the three differences are at positions (in MOR) Glu229 (in EL2), Lys303 (6.58) and Trp318 (7.35), which are Asp, Trp and Leu, respectively, in DOR. The substitution of leucine for tryptophan in DOR at position 7.35 is responsible for the binding selectivity both of the DOR-selective antagonist naltrindole and the conformationally constrained DOR-selective peptide agonist [D-Pen2-D-Pen5]enkephalin (DPDPE) [51]. Additionally, the Trp318Leu (7.35) mutation increased the affinity of both of these ligands at MOR [54].

Our conformational modeling studies of the hDOR-deltorphin complexes also suggest that position 7.35 is the key position in the binding pocket that defines the difference in selectivity between human and frog receptors (Fig. 6). In the human DOR, both deltorphin C and deltorphin II are predicted to bind with their Phe3 side chain optimally positioned in a hydrophobic pocket formed by Leu300(7.35), Val281(6.55) and Trp284(6.58), making a π-stacking interaction with the latter. In the frog DOR, the Trp at position 7.35 narrows the binding pocket and results in a severe steric clash with Phe3 of the deltorphin peptide that precludes optimal ligand binding. Note that the conformation of the mutant Trp7.35 in frog DOR is stabilized by contacts with nearby residues, such as a stacking interaction with Trp6.58, precluding optimal adjustment of the pocket and hampering accommodation of deltorphins. Two other non-conserved residues of DOR, Val197 (Thr in frog) and Arg190 (Lys in frog) in the ECL2 are in close proximity to the docked deltorphin and may interact with the ligand; however, such contacts in the more flexible and solvent-exposed part of the ligand are not predicted to significantly contribute to selectivity. This is in agreement with the observation that the Leu7.35Trp mutation in frog DOR almost completely rescues deltorphin binding, while the opposite mutation in the human receptor nearly eliminates deltorphin binding.

The molecular basis for the “species selectivity” of MOR-specific opioid like peptides from frog skin was also studied using chimeric receptors. The MOR-specific peptides tested in the current study were consistently more potent at the human than at the frog MOR. The sequences of the human and frog MORs differ in four regions, excluding the N- and C-termini. In studies using chimeric receptors, there was little or no effect of replacement of regions of the frog MOR with the human sequence, unless the N-terminus of the frog MOR was also replaced by the human sequence (Fig. 1B 5–7). This was not surprising, since the N-termini of these two receptors differ markedly, and since the N-terminus of MOR has been shown to be critical for ligand binding [55]. Once the N-termini had been swapped, the results showed that the human EL3 was important for receptor activation by all of the studied peptides (Table 3), and that the human EL2 had only a small effect on activation, except for dermorphin (Table 3 and Fig. 2A). Thus EL2, along with the N-terminus, may be considered to be a ‘specificity determinant’ for dermorphin at MOR. The binding of DAMGO and dermorphin to the rpMOR chimeras was well correlated with the response of the chimeric receptors in the functional assays; however, there is a discrepancy between the binding and function of DAMGO and dermorphin in wild type rpMOR. DAMGO and dermorphin are at least 100-fold less potent in rpMOR than in any of the h-rpMOR chimeras, but both peptides bind to rpMOR with affinity that is only slightly lower than that of h-rpMOR-WT and h-rpMOR-EL3h (Fig. 2A). This phenomenon cannot be attributed to

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low expression levels, since we have established comparable expression levels of human and frog receptors in HEK293 T cells (Table 1). It is conceivable that the N-terminus of MOR is involved in signaling or trafficking of the receptor, thus resulting in lower apparent receptor activity. The data presented here suggest that the low response of rpMOR to dermorphin and other peptides originates in the N-terminal region and in EL2.

The functional and binding data of the frog DOR and its humanized variants is more internally consistent than in the case of MOR, *i.e.*, a lower potency is reflected in a lower binding affinity. In this case, we discovered that the EL3 of the frog DOR has an important role in its "deltorphin insensitive" phenotype. Further analysis led to the discovery that Trp301(7.35) of the frog DOR is the critical residue for insensitivity to deltorphin (Fig. 2B, Table 3). This notion was further supported by an analysis of the reverse mutation in the human receptor, Leu300Trp. The data in Figs. 2B and 3C demonstrate that Leu300 (7.35) is a critical residue for activation of hDOR by DOR-specific agonists, especially in the case of the *Phyllomedusa*-derived peptide deltorphin (Fig. 4). Phylogenetic analysis of DOR orthologs revealed that all of the available sequences from LFA contain a tryptophan at position 7.35 (Fig. 3A and B). Together, these data lead to the prediction that LFA are deltorphin-insensitive. This distinct functional difference between DORs from terrestrial vertebrates and non-mammalian aquatic vertebrates and amphibians (LFA) leads to a hypothesis that the increased sensitivity and selectivity of DOR have evolved, and been maintained, after divergence of amphibians and mammalians.

To test this "aquatic deltorphin insensitivity" hypothesis, we chose the zebrafish model system as an independent model; unlike enkephalins, deltorphins did not have any effect in the zebrafish swimming assay (Fig. 5). It is important to note that binding assays done with *Rana pipiens* tissue demonstrated that the affinities of deltorphin and dermorphin to ORs in the brains and spinal cord are very low, (30–80 μM) [56, 57] and, although these peptides have been suggested to have some *in-vivo* effects in *Rana pipiens* [58], it seems unlikely that these results are due to interactions with opioid receptors as they do not bind to them with a pharmacologically relevant affinity.

The skin secretions of frogs, toads and other amphibians contain a multitude of psychoactive compounds to which they may not be sensitive. Here this concept is demonstrated for opioid peptides, and it may be the case for other psychoactive alkaloids and peptides secreted by amphibians. The skin secretions of the toad *Bufo alvarius* contain, among other substances, the psychoactive compounds 5-Me-DMT and 5-OH-DMT; these compounds primarily target serotonin receptors, and they can generate different serotonin-like effects, including hallucinations [59]. Bombesin, bradykinin and tachykinin peptides are also found in the skin secretions of amphibians [41], and we hypothesize that the amphibian orthologs of the receptors for these compounds are likely to be insensitive to them. We are currently testing this hypothesis with a large number of other amphibian GPCR-skin secretants to determine if there is a generalized mechanism for this insensitivity related to differential receptor sensitivity.

It is interesting to consider the evolutionary advantages of these systems. Thus, for evolutionary success, the molecular structure of toxin target sites (*e.g.*, receptors) should be

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conserved in a phylogenetically wide spectrum of taxa [45]. Toxin inactivation or receptor insensitivity seems to be related to the development of such poisons. How ORs and opioid-like peptides might have evolved in frogs remains a matter for speculation, although our data suggest that FLA maintained the deltorphin insensitive phenotype (Trp at position 7.35) even though there is no evidence that they secrete or come in contact with deltorphins in their natural habitats [60]. *Phyllomedusa* spp. (the source of the skin peptides) are considered by amphibian biologists the newest frog species [61, 62], suggesting that these peptides are a more recent evolutionary trait. Together these data suggest that the lower type selectivity hypothesized for opioid receptors in FLA, might have been important for the ability of later amphibian species to develop the highly potent opioid like peptides in their skin secretions.

Significance

Comparative pharmacology is useful for exploring questions related to structure function relationship and evolutionary mechanisms. Profound pharmacological distinctions along with a well-defined sequence differences between human and frog receptors allowed the identification of the molecular basis for an interesting evolutionary phenomena. It has been previously suggested that the evolution of vertebrate opioid receptors (ORs) followed a vector of increasing type selectivity and support this idea and show that as general rule opioid peptides activate human receptors to a higher degree than the frog receptors and the tested peptides exhibited increased type selectivity in the human receptors relative to the frog receptors. Interestingly, some of the most potent opioid peptides, known, have been isolated from amphibian skin secretions. Here we show that such peptides (dermorphin and deltorphin), while highly potent in the human receptors and inactive in frog ORs. The molecular basis for the insensitivity of the frog ORs to these peptides was studied using chimeras and molecular modeling. While the molecular mechanism for the insensitivity was not completely resolved due to its complexity, the delta opioid receptor (DOR) insensitivity to deltorphin was shown to be due to variation of a single amino acid– Trp7.35—which is a Leu in mammalian DORs. Notably, Trp7.35 is completely conserved in all known DOR sequences from lamprey, fish and amphibians. The deltorphin-insensitive phenotype was verified in zebra fish. Our results provide a molecular explanation for the species selectivity of skin-derived opioid peptides and raise an interesting discussion on the potential part that the low selectivity of frog ORs might have had in the development of such peptides.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection

Human embryonic kidney (HEK) 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Corning) supplemented with 10% fetal bovine serum, 10 U/mL penicillin and 10 μg/mL streptomycin (Gibco). The cells were grown in a humidified incubator in the presence of 5% CO_2 at 37 °C. Transient transfection (48 h) of wild-type, chimeric, and mutant receptor cDNAs was performed in 15-cm tissue culture plates using an optimized calcium phosphate method [63].

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Construction of Chimeric and Mutant Receptors

Human opioid receptor cDNAs in the mammalian expression vector pcDNA3.1(+) (Invitrogen) were obtained from the UMR cDNA Resource Center (www.cdna.org). Opioid receptors from *Rana pipiens* had previously been subcloned into the same vector [6]. Site-directed mutagenesis was done using the QuikChange II® mutagenesis kit (Agilent) according to the manufacturer's instructions, and all mutations were confirmed by full-length automated DNA sequencing (Eton Bioscience, Durham, NC).

Generation of inter-species MOR and DOR chimeras was done using a two-step overlap PCR method, as previously described [64], and as diagrammed in Supplementary Fig. 2. The sequences of all primers used are given in Supplementary Table 1. A total of 5 DOR chimeras (Fig. 1C) and 7 MOR chimeras (Fig. 1B) was made by "humanizing" regions of the frog receptors (*i.e.*, replacing stretches of residues from the frog receptors with their human counterparts).

In vitro characterization of OR activation

Radioligand Binding Assay—Radioligand binding assays were performed as previously detailed [65] using ^{3}H-diprenorphine (Perkin Elmer) for saturation binding assays (10–0.1 nM), with 10 μM unlabeled naltrexone (Sigma) to determine nonspecific binding.

Functional assay—Inhibition of cAMP production was measured using the genetically encoded cAMP biosensor, Glosensor-22F (Promega) as previously described [66].

Modeling of DOR-deltorphin interactions

Docking of opioid peptides was performed with the hDOR structure recently solved in complex with DIPP-NH_2 peptide solved at 2.5 Å resolution (PDB: 4RWD) [39]. The receptor model was protonated and prepared using an ICM docking pipeline. Three models were tested, one with all 23 water molecules retained from the crystal structure, one without waters, and one with only the three water molecules mediating ligand interactions in hDOR-DIPP-NH_2 retained. These three waters were also found to be conserved among the structures of different opioid receptor types.

Fully flexible models of the heptapeptides deltorphin C (Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2) and deltorphin II (Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2) were docked into the hDOR model using the ICM energy-based docking algorithm, which takes advantage of the internal molecular coordinates of the ligand. A settings of thoroughness=30 was used to ensure exhaustive sampling of the peptide conformational space within the binding pocket. A large docking box (25×25×25 Å) was used to ensure that it completely covers the peptide binding pocket and the extracellular entrance of the receptor.

Ligand poses with the best binding scores were clustered and further analyzed using all atom optimization with side chain flexibility in the ligand binding pocket. Final energy-based binding scores were calculated using the ICM scoring algorithm. The mutation Leu7.35Trp was introduced by replacing the Leu7.35 side chain with the Trp side chain in ICM, followed by thorough energy-based optimization of the adjacent residues. The peptides were

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docked in the mutated receptor, and binding scores calculated. In addition, the conformational preferences of the free deltorphin peptides in solvent were studied using exhaustive sampling of their rotamers, and the resulting low-energy conformations were compared to the docked deltorphins.

In vivo activity of DOR agonists in zebrafish

Aquaculture—We collected fertilized eggs from group matings of Ekkwill strain zebrafish (Ekkwill Waterlife Resources, Ruskin, FL). Embryos were raised in HEPES (10 mM, pH7.2) buffered E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$ and 0.33 mM $MgSO_4$) on a normal 14/10 h on/off light cycle at 28 °C. At 3 days post-fertilization (dpf), chorion debris was removed and larvae were transferred into fresh medium until 7 dpf. At 7 dpf, larvae were counted and manually pipetted, 10 larvae per well, into clear 96-well, U-bottom plates (250μl/well) in the same medium.

Peptide treatment—All peptides were dissolved in DMSO, diluted to a 100X concentration and added directly to zebrafish medium in 96-well plates (2.5 μl in 250 μl). Wells were mixed and allowed to incubate for 1 h at 28 °C in ambient light prior to behavioral evaluation. The final DMSO concentration was 1%.

Swimming Assay—96-well plates were loaded into a ZebraBox containing a computer-controlled light box and a video camera with an infrared filter (ViewPoint Life Sciences, Montreal, Quebec, Canada). Infrared light was used to illuminate the chamber and the temperature was maintained at 28 °C for the duration of the experiment. White light stimulation was automated using ZebraLab software (ViewPoint Life Sciences) as follows: dark for minutes 1, 3, 5 and 7; light for minutes 2, 4 and 6. At this age, darkness stimulates fish swimming [67]. Locomotor activity was recorded during the dark phases by the infrared camera using the ZebraLab Videotrack quantization mode at 30 frames/second. 96 evenly-spaced regions of equal size were drawn around each well of the assay plate using the Viewpoint software. The software tracks the change in pixel intensity for each region over time producing a motion index, which correlates with the overall amount of motion in the well. Each video was saved for review and the data were further analyzed using custom R scripts. We used a Student's t-test (2-tailed, unpaired, unequal variance) to analyze motion index values. An effect was considered significant if $p<0.05$. All zebrafish protocols were approved by the Institutional Animal Care and Use Committee at Massachusetts General Hospital.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

1. Comparison of frog and human opioid receptors show distinct pharmacology
2. Frog-derived opioid peptides secreted are potent in human but inactive in frogs
3. The molecular basis for δ-receptor species selectivity due to a single amino acid
4. The structural basis for this selectivity filter is elucidated

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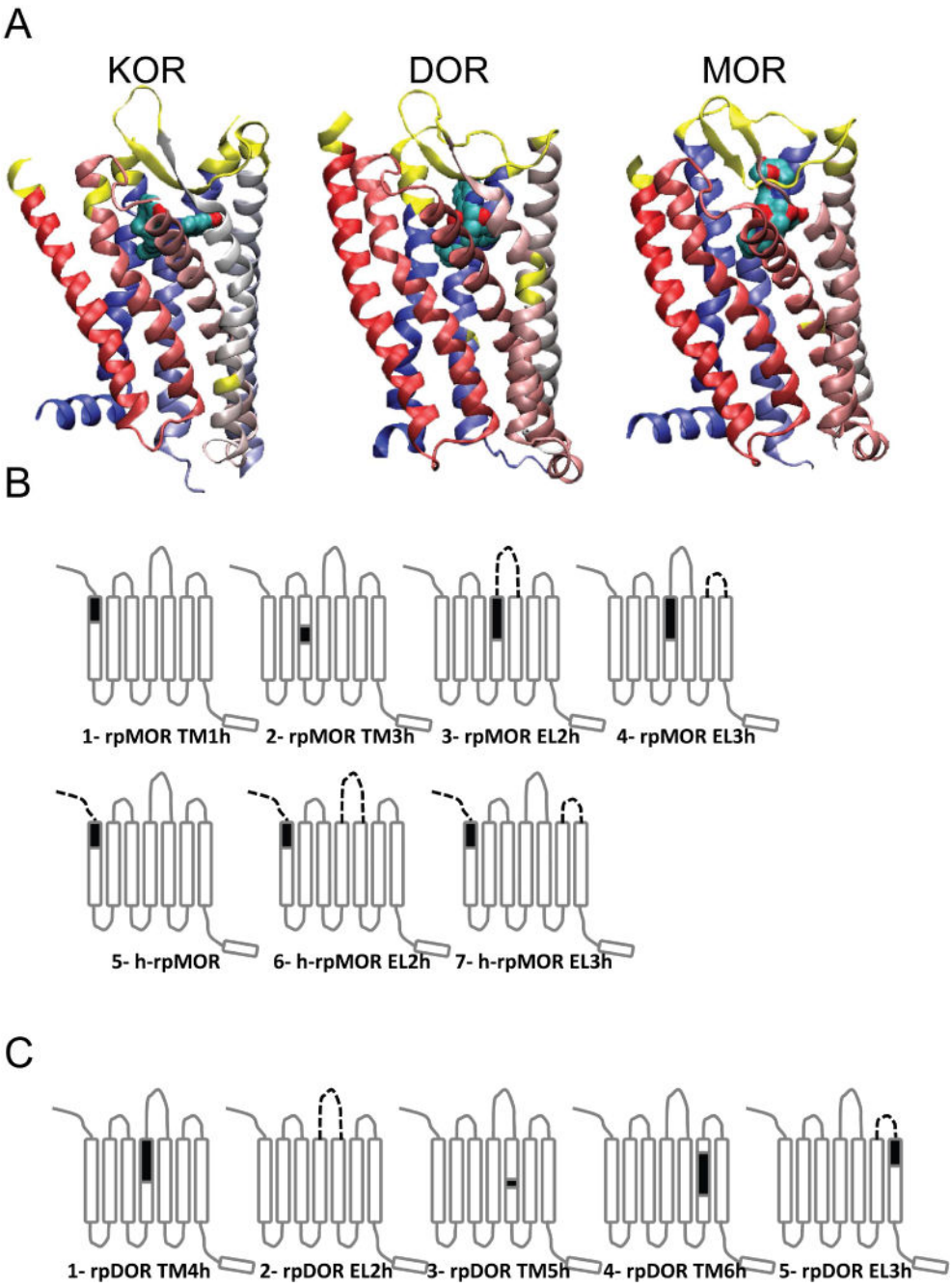

Figure 1. Sequence divergence in ORs from frogs and humans

A- Crystal structures of the human KOR DOR and MOR. Transmembrane domains are colored using a color gradient from red (TM1) to blue (TM7), yellow regions are the receptor regions that differ between human and frog receptors (for more details, see sequence alignments in Fig. S1). **B and C**- Chimeras used in this study. rpDOR chimeras (B1–5) and rpMOR chimeras (C1–7). The chimeras were constructed by replacing regions of rpOR (white with grey outline) with their human corresponding sequences (black).

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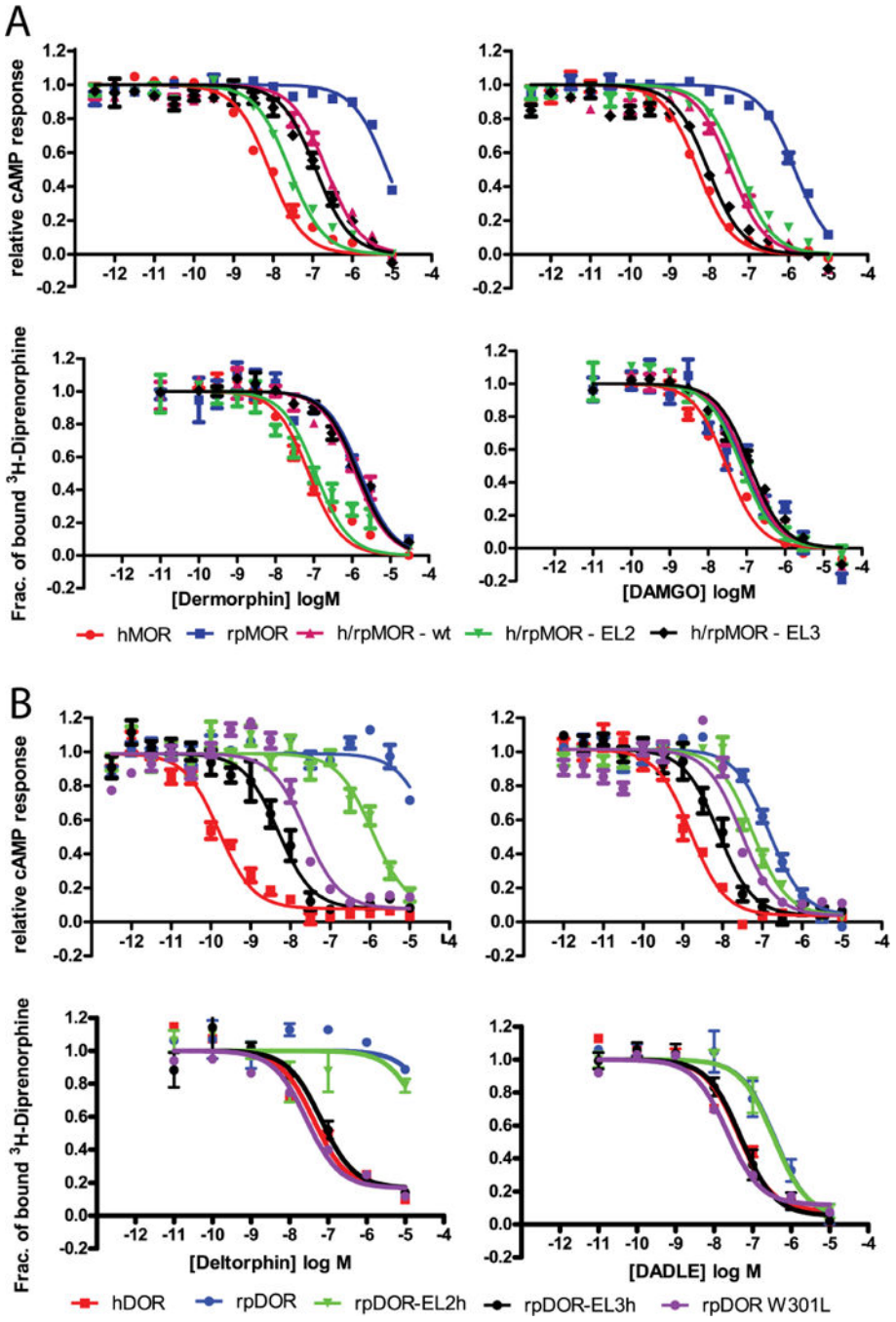

Figure 2. Molecular basis for dermorphin and deltorphin insensitivity in rpORs

Inhibition of isoproterenol (300 nM) induced cAMP response and [^{3}H]-diprenorphine competition in MOR variants by dermorphin and DAMGO (hMOR ●, rpMOR ■, h-rpMOR WT ▲, h-rpMOR – EL2h ▼, h-rpMOR – EL3h ◆) (A) and in DOR variants by deltorphin and DADLE (hDOR ■, rpDOR ●, rpDOR EL2h ●, rpDOR – EL2h ▲, rpDOR – EL3h ●) (B).

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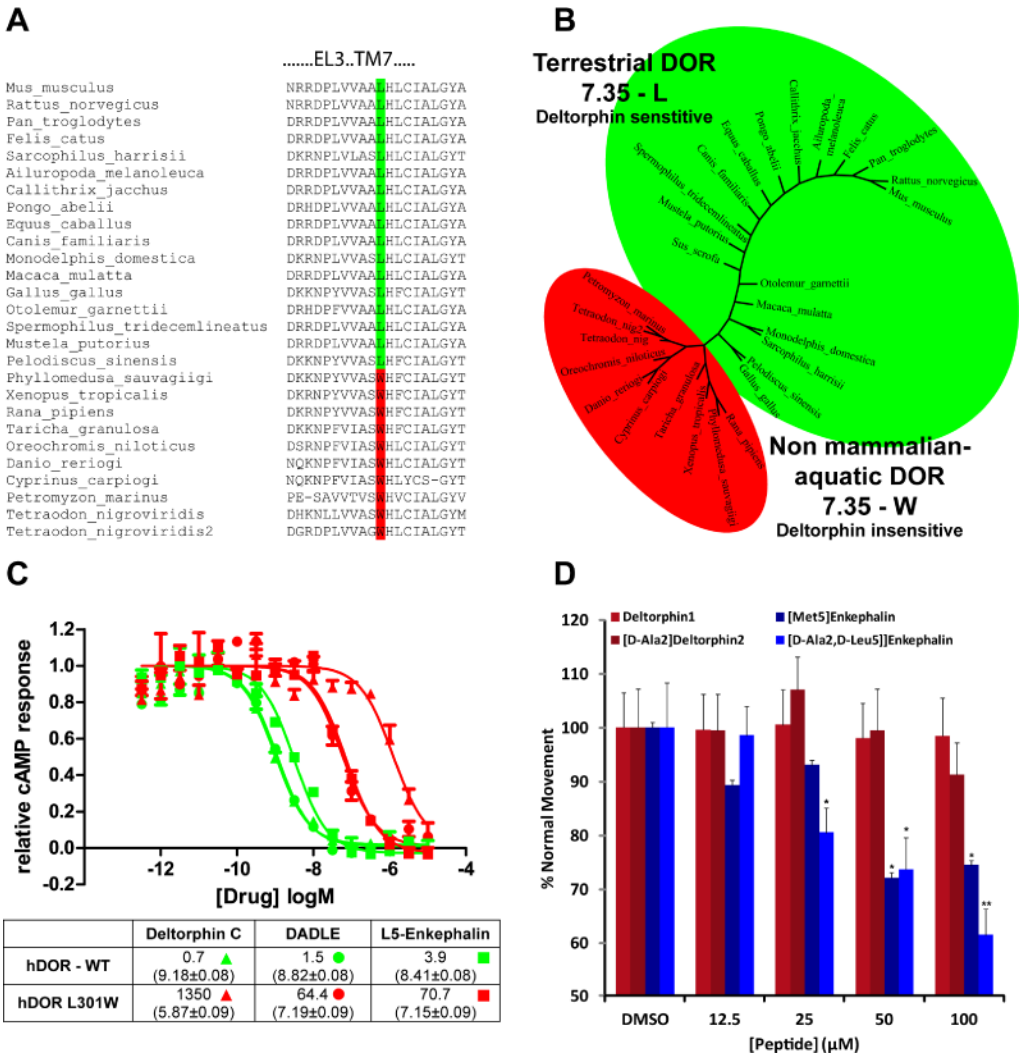

	Deltorphin C	DADLE	L5-Enkephalin
hDOR - WT	0.7 ▲ (9.18±0.08)	1.5 ● (8.82±0.08)	3.9 ■ (8.41±0.08)
hDOR L301W	1350 ▲ (5.87±0.09)	64.4 ● (7.19±0.09)	70.7 ■ (7.15±0.09)

Figure 3. Position 7.35 in DOR is the selectivity filter

A- comparison of DOR EL3-TM7 sequence from different species highlighting position 7.35 with yellow (L) and green (W). B- Phylogenetic tree of vertebrate delta opioid receptors (full sequence) DOR from terrestrial vertebrates (yellow background) contains leucine at position 7.35 and DOR from non-terrestrial vertebrates (green background) contains tryptophan at position 7.35. C-The effect of tryptophan at position 7.35 in human DOR. Inhibition of cAMP response by three peptides in cells expressing hDOR or hDOR Leu300Trp. D-Zebrafish are sensitive to enkephalins but not deltorphins. Zebrafish larvae (7 dpf) were treated with increasing concentrations of DOR peptide ligands for 1 h and then assayed for normal swimming activity. Results represent average motion ±SEM from 12 replicate wells of 10 fish each, normalized to the vehicle (DMSO) control. Statistical analysis was performed using Student's t-test (2-tailed, unpaired, unequal variance) significance was determined as follows: *p<0.05; **p<0.01.

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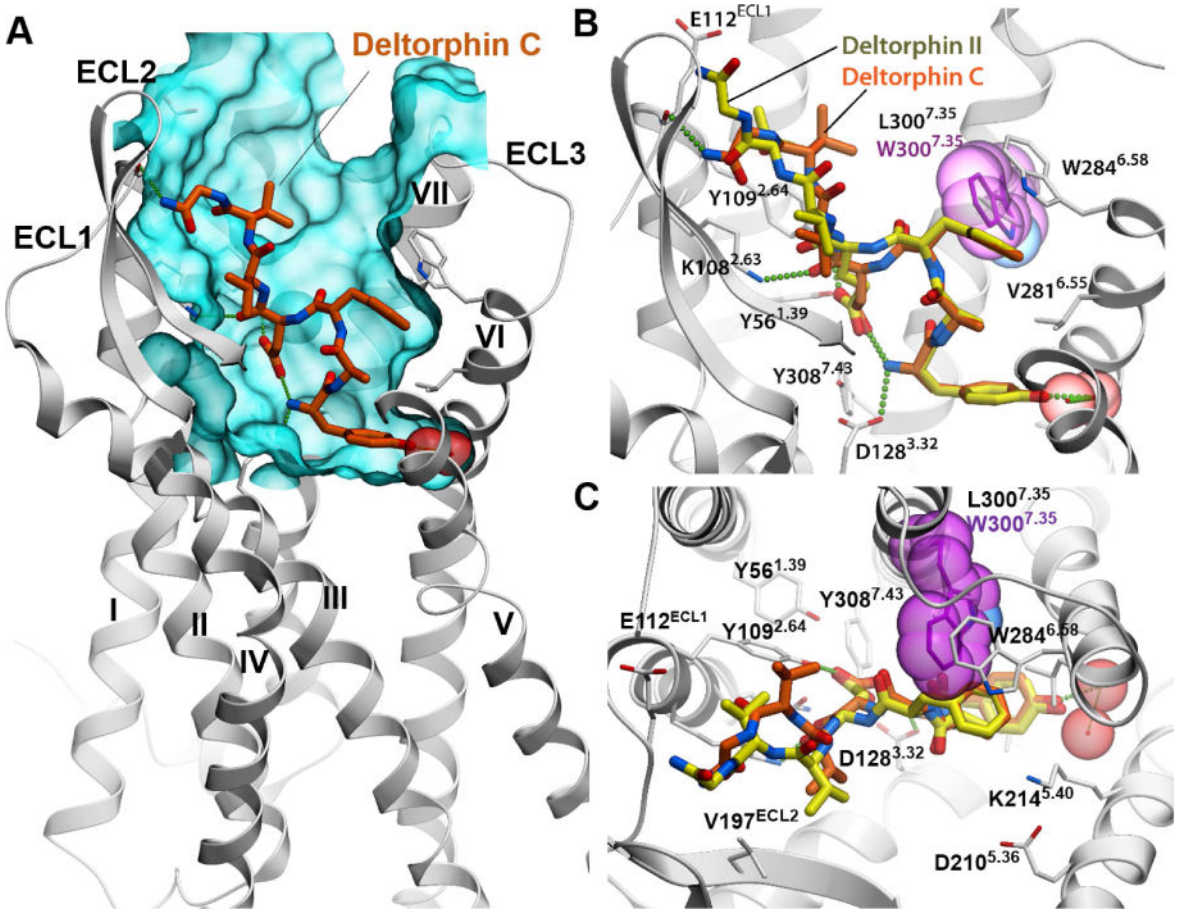

Figure 4. Structural model of Deltorphin and DOR

A- Surface representation of the binding pocket (cyan) of the delta opioid receptor and the docked position of Deltorphin C (orange). B and C –A Trp at position 7.35 of the hDOR Leu300Trp mutant protrudes into the binding pocket and narrows the entrance to the binding pocket. Deltorphin C (orange carbon atoms) and deltorphin II (yellow carbon atoms), docked into hDOR structure are shown in stick presentation. The Trp7.35 side chain in rpDOR (shown with magenta carbons in both stick and transparent sphere presentation) clashes with deltorphin peptides and can occlude their binding.

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Table 1

[^{3}H]Diprenorphine saturation binding

Receptor expression levels (B_{max}) and affinity (K_D) for diprenorphine (Data expressed as mean ± SEM).

	KOR		MOR		DOR	
	Frog	**Human**	**Frog**	**Human**	**Frog**	**Human**
B_{max} (pMol/mg)	2 ± 0.2	2.6 ± 1.2	8.8 ± 1.3	2.2 ± 0.3	2.1 ± 0.3	5.7 ± 1.1
K_D (nM)	1.2 ± 0.3	0.8 ± 0.6	2.2 ± 0.9	0.6 ± 0.2	0.8 ± 0.3	1.8 ± 0.9

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Table 2

Comparative pharmacology of opioid receptors from *Homo sapiens* and *Rana pipiens*

Activation of opioid receptors by different opioid peptides was measured by inhibition of isoproterenol-induced cAMP production in HEK 293T cells. Ligand potency in each receptor is presented $EC50_{nM}$ ($EC50_{logM}$ ±SEM); data is average of at least three separate experiments.

	KOR		MOR		DOR	
	Human	**Frog**	**Human**	**Frog**	**Human**	**Frog**
Deltorphin II	NA	NA	2700 (−5.6±0.07)	NA	1 (−9.07±0.05)	NA
DADLE	3100 (−5.5±0.16)	NA	14 (−7.86±0.03)	480 (−6.3±0.05)	4 (−8.4±0.06)	353 (−6.5±0.06)
DAMGO	1800 (−5.7±0.13)	2400 (−5.6±0.09)	4.9 (−8.3±0.03)	1500 (−5.8±0.07)	NA	NA
Deltorphin C	NA	NA	620 (−6.2±0.05)	NA	0.4 (−9.36±0.07)	NA
Dermorphin	NA	NA	6.5 (−8.2±0.05)	NA	NA	NA
Dynorphin A	3.8 (−8.4±0.08)	33 (−7.5±0.06)	1000 (−6.0±0.08)	4800 (−5.3±0.12)	370 (−6.4±−0.09)	1700 (−5.8±0.09)
Dynorphin B	25 (−7.6±0.06)	170 (−6.8±0.05)	900 (−6.1±0.09)	5200 (−5.3±0.16)	360 (−6.4±0.15)	5200 (−5.3±0.17)
Endomorphin 1	NA	NA	20 (−7.71±0.07)	381 (−6.4±0.07)	NA	NA
Endomorphin 2	NA	NA	42 (−7.4±0.10)	782 (−6.1±0.06)	NA	NA
L5-Enkephalin	2500 (−5.6±0.09)	NA	1500 (−5.9±0.09)	NA	54 (−7.3±0.08)	1800 (−5.8±0.07)
M5-Enkephalin	611 (−6.2±0.08)	NA	140 (−6.9±0.13)	2200 (−5.7±0.2)	37 (−7.4±0.09)	180 (−6.8±0.08)
Salvinorin A	0.9 (−9.1±0.07)	69 (−7.16±0.08)	NA	NA	NA	NA
α-Neoendorphin	11 (−8.0±0.08)	78 (−7.1±0.09)	NA	NA	1600 (−5.8±0.08)	1600 (−5.8±0.08)
Xendorphin	26 (−7.6±0.10)	33 (−7.5±0.07)	1400 (−5.9±0.12)	NA	NA	NA

NA – not active

Data are expressed in EC_{50}(nM) and (log EC_{50} (M) ±SEM).

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Table 3

Effect of chimeras with humanized N-terminal on the function of rpMOR

Activation of chimeric MOR variants by different opioid peptides was measured by inhibition of isoproterenol-stimulated cAMP response in HEK 293T cells. Ligand potency in each receptor is presented (average of at least three separate experiments).

	hMOR	rpMOR	h-rpMOR-wt	h-rpMOR-E2h	h-rpMOR-E3h
DAMGO	11 (−8.0 ± 0.08)	1900 (−5.7 ± 0.2)	96 (−7.0 ± 0.08)	81 (−7.1 ± 0.07)	22 (−7.7 ± 0.07)
	K_i=30nM	K_i=160nM	K_i=100nM	K_i=80nM	K_i=120nM
Dermorphin	5.9 (−8.2 ± 0.07)	NA	230 (−6.7 ± 0.08)	40 (−7.4 ± 0.07)	130 (−6.9 ± 0.1)
	K_i=50nM	K_i=1640nM	K_i=1230nM	K_i=112nM	K_i=1410nM
Endomorphin 1	14 (−7.9 ± 0.08)	360 (−6.5 ± 0.1)	43(−7.4 ± 0.07)	95 (− 7.0 ± 0.06)	14 (−7.9 ± 0.06)
Endomorphin 2	62 (−7.1 ± 0.08)	920 (−6.0 ± 0.12)	93 (−7.0 ± 0.0.27)	190 (−6.7 ± 0.32)	32 (−7.5 ± 0.0.31)
Dynorphin A	1300 (−5.9 ± 0.16)	5100 (−5.3 ± 0.14)	1300 (−5.9 ± 0.0.27)	2500 (−5.6 ± 0.17)	520 (−6.3 ± 0.22)

NA not active

Data are expressed in EC_{50}(nM) and (log EC_{50} (M) ±SEM).