The Evolution of Opioid/Orphanin Receptors in Chordates

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The Evolution of Opioid/Orphanin Receptors in Chordates

A Thesis
Presented to
The Faculty of Natural Sciences and Mathematics
University of Denver

In Partial Fulfillment
of the Requirements for the Degree
Master of Science

by

Jazalle D. McClendon

November 2009
Advisor: Robert M. Dores
Abstract

The interest in the opioid/orphanin gene family stems from functional similarities that these peptides have to the narcotic opium. Opiates have been extensively studied because of their analgesic properties; however, the reason that these plant products can affect the human central nervous system was a mystery until the discovery of opiate-like peptides. The endogenous opioid peptides are well understood today because they have been fully cloned and characterized in several different organisms including lower chordates. On the other hand, the opioid/orphanin receptors have not been fully cloned or characterized in lower chordates; therefore, to better understand the past and present evolutionary path of the opioid/orphanin gene family it is important to have opioid receptor sequence information available over a broad taxonomic scale in lower chordates. This study reveals opioid/orphanin receptor sequence from Petromyzon marinus (lamprey), Heterodontus francisci (horn shark), and Acipenser transmontanus (white sturgeon). This sequence information exposed the conservation of critical amino acids within the opioid binding pocket and disulfide bridge, uncovered the possibility of crucial amino acids involved in ligand selectivity, and offered a proposed hypothesis for the evolutionary relationship between the four opioid/orphanin receptors.
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Introduction

1. Discovery of Opiates

The interest in the opioid/orphanin gene family stems from functional similarities that these peptides have to the narcotic opium. Opium is an alkaloid derived from the opium poppy *Papaver somniferum* L.; a colorful flower harvested all over the world (Corbett et al., 2009). There are many alkaloid constituents that can be extracted from the opium poppy including morphine, noscapine, codeine, and thebaine. Opiates have been extensively studied because of their analgesic properties; however, the reason that these plant products can affect the human central nervous system was a mystery (Aggrawal, 1995) until the discovery of opiate-like peptides (Hughes et al., 1975).

2. Overview of Opioid Ligands and their Precursors

The first opioid peptides isolated from pig brain were the enkephalins. They are composed of two pentapeptides with amino acid residues H-Tyr-Gly-Gly-Phe-Met-OH (YGGFM) and H-Tyr-Gly-Gly-Phe-Leu-OH (YGGFL). These peptides were termed Methionine-enkephalin (Met-Enk) and Leucine-enkephalin (Leu-Enk), respectively (Hughes et al., 1975). Larger opioid peptides were later discovered that contained either a Met-Enk or Leu-Enk sequence as their core fragment (Kakidani et al., 1982). Isolated from camel pituitary extracts, β-endorphin (β-End) is a C-terminally extended form of Met-Enk with an additional 26 amino acids at the carboxyl end (Table 1) (Li and Chung,
As the isolation of dynorphins began, two more hypothalamic opioid peptides with Leu-Enk core fragments at their amino-terminals were revealed; a decapeptide termed \(\alpha\)-neoendorphin with a five amino acid carboxy-terminal extension and a nonapeptide termed \(\beta\)-neoendorphin with a four amino acid carboxy-terminal extension. (Table 1) (Kangawa et al. 1979, 1981; Minamino et al., 1981). Finally two additional opioid peptides isolated from porcine and bovine pituitary were discovered; Dynorphin A (Dyn A) a heptadecapeptide with a Leu-Enk core fragment and 12 amino acid carboxy-terminal extension (Goldstein et al., 1979 & 1981) and Dynorphin B (Dyn B) a tridecapeptide with a Leu-Enk core fragment and an eight amino acid carboxy terminal extension (Table 1) (Fischli et al., 1982; Kilpatrick et al., 1982)

These biologically active opioid peptides were found to be derivatives of larger protein precursors due to post-translational processing (Hughes et al., 1980). This post-translational processing was thought to involve trypsin-like proteolytic cleavage at characteristic sites with paired, basic amino acids that flank the biologically active opioid sequence as shown in Figure 2 (Steiner et al., 1974; Steiner, 1976). Subsequent studies implicated their serine proteases prohormone convertase I and prohormone convertase II as the enzymes involved in the endoproteolytic cleavage of opioid precursors (Seidah et al., 1999). It has now been shown that the opioid peptides are encoded by three distinct prohormone precursors, proenkephalin (Pro-Enk), proopiomelanocortin (POMC), and prodynorphin (Pro-Dyn). Pro-Enk cDNA’s were cloned by Noda et. al (1982) revealing one Leu-Enk and four Met-Enk sequences. Cleavage of POMC has been shown to produce \(\beta\)-End, adrenocorticotropic hormone (ACTH), and \(\alpha\)-melanocyte stimulating hormone (\(\alpha\)-MSH) (Nakanishi et al., 1979). Pro-Dyn cleavage produces dynorphin A,
dynorphin B, α-neoendorphin and β-neoendorphin (reviewed in Dores et al., 1993).

Figure 1 shows the precursors and the active peptides that they produce.
### Amino Acid Sequences of Opioid Peptides

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methionine-enkephalin (Met-Enk)</td>
<td>YGGFM</td>
</tr>
<tr>
<td>Leucine-enkephalin (Leu-Enk)</td>
<td>YGGFL</td>
</tr>
<tr>
<td>β-endorphin (β-End)</td>
<td>YGGFM TSEKSQTPLVTLFKNAIVKNAHKKGQ</td>
</tr>
<tr>
<td>α-neoendorphin (α-neoend)</td>
<td>YGGFLRKYP</td>
</tr>
<tr>
<td>β-neoendorphin (β-neoend)</td>
<td>YGGFLRKYP</td>
</tr>
<tr>
<td>Dynorphin A (Dyn A)</td>
<td>YGGFLRRIRPKKLKWDNQ</td>
</tr>
<tr>
<td>Dynorphin B (Dyn B)</td>
<td>YGGFLRRQFKKVVT</td>
</tr>
<tr>
<td>Nociceptin/Orphanin FQ (N/OFQ)</td>
<td>FGGFTGARKSARKLANQ</td>
</tr>
</tbody>
</table>

**Table 1.** Amino acid sequence of each opioid peptide discussed in the text. The italicized letters represent the core fragment for each peptide. The bold letter represents a Methionine (M) core fragment or a Leucine (L) core fragment (Hughes et al., 1980).
Figure 1. Structure of Precursor Proteins

Schematic structure of each precursor gene with their characteristic cysteine-rich amino-terminus and paired, basic amino acids which flank the biologically active opioid sequence (Dores et al, 1993). The line above α-neoendorphin represents amino acid residues 1-10 while the line above β-neoendorphin represents amino acid residues 1-9 (refer to Table 1).
3. Opioid Receptors in Mammals

In the early 1970’s, almost in parallel to the discovery of endogenous opioid ligands, binding studies using radiolabelled opioid ligands revealed the presence of opioid receptors (Pert and Snyder, 1973; Simon et al., 1973; Terenius, 1973). Martin and colleagues proposed that there were three receptors types based on studies that showed different pharmacological effects in response to several morphine- and nalorphine-like drugs administered to the chronic spinal dog. The receptors were named according to the drug that elicited the syndrome; Morphine interacted with the mu (µ) opioid receptor (MOR), Ketocyclazocine interacted with the kappa (κ) opioid receptor (KOR), and SKF-10,047 (N-allylnormetazocine) interacted with the sigma (σ) opioid receptor (Martin et. al, 1976; Gilbert et. al, 1976). It was later shown that the σ-opioid receptor was not a true opioid receptor because the opioid antagonist naloxone was unsuccessful in antagonizing the binding and in vivo effects of N-allylnormetazocine and related benzomorphan alkaloids (Mannalack et al., 1986). In 1977 Lord and colleagues discovered that the action of the enkephalins in mouse vas deferens acted on a different receptor than the receptors that morphine and morphine-like agonists act because 10 times more naloxone was required for the successful antagonism of enkephalins than for that of normorphine; this new receptor was designated delta (δ) opioid receptor (DOR) (Lord et al., 1977; Lord et al., 1976). Therefore, the three classic opioid receptors presently found in mammals are MOR, KOR, and DOR.

Although these three classic receptors can bind to several different endogenous opioid ligands produced by the three prohormone precursors mentioned earlier, they also have specific ligands that they bind to preferentially. Ligand selectivity is based on the
post-translational processing of the peptide revealing that two opioid recognition cores are responsible for binding the three receptors MOR, DOR, and KOR. The YGGFM or YGGFL core is required and adequate for binding DOR and MOR while an arginine extension to this core, YGGFM_R or YGGFL_R, is required and adequate for KOR binding. The shorter Pro-Enk products bind with the greatest affinity to DOR, but the C-terminally extended enkephalins bind to all three receptors with a high affinity. POMC products such as β-endorphin have an equal affinity for both DOR and MOR but low affinity for KOR. KOR demonstrates the maximum level of ligand selectivity of all three receptors binding with the highest affinity to Pro-Dyn products. However Pro-Dyn products can also bind to MOR and DOR (Mansour et al., 1995). Figure 3 demonstrates this receptor/ligand binding relationship for all three receptors.
**Table 2. Receptor/Ligand Binding Relationships**

Arrows represent receptor/ligand binding with an upward arrow (↑) symbolizing strong/high affinity, a downward arrow (↓) symbolizing poor/low affinity, and no arrow symbolizing no receptor/ligand binding. C-terminally extended enkephalins bind MOR, DOR, and KOR with high affinity, but do not bind ORL-1. Although dynorphins bind MOR and DOR, they bind KOR with the greatest affinity and do not bind ORL-1 at all. β-endorphin binds MOR and DOR with equal affinity, KOR with low affinity, and does not bind ORL-1. Orphanin FQ binds only to ORL-1 (Mansour *et al.*, 1995). *Kd* values for these receptor subtypes can be obtained from the Mansour *et al* (1995) paper.
In the search for other members of the opioid receptor gene family, Mollereau and colleagues isolated an orphan receptor from a human cDNA library encoding Opioid Receptor Like 1 (ORL-1). The receptor protein consisted of 370 amino acids and demonstrated high sequence identity to that of the other three classical opioid receptors (Mollereau et al., 1994). This same receptor was later independently isolated from murine clones (Bunzow et al., 1994; Fukuda et al., 1994; Lachowicz et al., 1995; Nishi et al., 1994; Wang et al., 1994; Wick et al., 1994) and showed ~65% sequence homology to each of the other three opioid receptors. This is consistent with the sequence homology between any of the two classical opioid receptors which is about 70% in mammals (Chen et al., 1994). The pharmacological effects exerted by this newly discovered orphan receptor did not resemble any of the effects exerted by the previously isolated endogenous opioid ligands or any of the opioid receptor agonists; this led two separate groups to isolate the endogenous ligand for ORL-1. Using reverse pharmacology (Libert, F. et al., 1991), based on the identification of the ORL-1 receptor, they discovered a heptadecapeptide called nociceptin/orphanin FQ (N/OFQ) that closely resembled dynorphin A with the amino acid sequence FGGFTGARKSARKLANQ (Table 1) (Meunier et al. 1995; Reinscheid et al., 1995). The partial cDNA isolated from rat-brain library encodes this ligand flanked by Lys-Arg proteolytic cleavage motif which is consistent with the other opioid ligands described earlier (Meunier et al., 1995). This partial cDNA sequence suggests that N/OFQ is processed from a larger protein precursor just as the previously described opioid ligands were. This precursor protein named prepronociceptin (PPNOC) was later fully sequenced and characterized from human,
mouse, and rat; it was found to be most similar to POMC in that PPNOC encodes a single copy of N/OFQ (Mollereau et al., 1996). The discovery of ORL-1, its endogenous ligand, and the complete protein precursor, from which it is biologically processed, comprises the opioid/orphanin gene family. Molecular cloning of cDNA’s for each of the four receptors has shed light on their pharmacology, distribution, and structure.

3.1 Structural Characteristics of Opioid Receptors

Hydropathy analyses of the cloned receptor sequences revealed that opioid receptors are G-protein coupled receptors (GPCR’s) characterized by seven transmembrane-spanning domains (Figure 2). In mammals, the receptors show the highest sequence identities to each other within the transmembrane regions (41%-76%) and intracellular regions (59%-89%). The extracellular regions are the most diverse sharing only 6%-7% sequence identity with the exception of the first extracellular loop (Table 3). The formation of a disulfide bridge between cysteine residues in the first and second extracellular loop seems to be essential in stabilizing the functional receptor protein structure. Increasing levels of reducing agents such as dithiothreitol (DTT) break down disulfide bridges and cause the opioid receptor to become increasingly more asymmetrical which inhibits opioid agonists from binding to the receptor molecule (Gioanniani et al., 1989; Fawzi et al., 1997). Another structural characteristic of opioid receptors is N-linked glycosylation sites within the N-terminal domain; each receptor has anywhere from two to five glycosylation sites within its N-terminus (reviewed in Satoh & Minami, 1995) (Figure 2). These glycosylation sites could be important in the binding affinity of specific endogenous opioid ligands (Kieffer et al., 1992).
Figure 2: Structure of Opioid Receptors

Opioid receptors have seven transmembrane-spanning (TM1-7) domains within the plasma membrane, three extracellular loops (EL 1-3) on the N-terminus side, and three intracellular loops (IL 1-3) on the C-terminus side. They are most highly conserved within the TM regions and the IL regions. They show high diversity within the EL region. The disulfide bridge between the cysteines (red circles) in the first and second extracellular loop is consistent between all four receptors. The green dots represent potential N-linked glycosylation sites within the four opioid receptors (Satoh & Minami, 1995).
### Amino Acid Sequence Identity Among Human, Rat, and Mouse Opioid Receptors

<table>
<thead>
<tr>
<th></th>
<th>Amino Acid % Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>hOR</td>
</tr>
<tr>
<td><strong>Extracellular (EC) Regions</strong></td>
<td></td>
</tr>
<tr>
<td>1st EC Loop</td>
<td>50</td>
</tr>
<tr>
<td>2nd EC Loop</td>
<td>7</td>
</tr>
<tr>
<td>3rd EC Loop</td>
<td>6</td>
</tr>
<tr>
<td><strong>Transmembrane (TM) Regions</strong></td>
<td></td>
</tr>
<tr>
<td>TM1</td>
<td>42</td>
</tr>
<tr>
<td>TM2</td>
<td>74</td>
</tr>
<tr>
<td>TM3</td>
<td>73</td>
</tr>
<tr>
<td>TM4</td>
<td>23</td>
</tr>
<tr>
<td>TM5</td>
<td>54</td>
</tr>
<tr>
<td>TM6</td>
<td>41</td>
</tr>
<tr>
<td>TM7</td>
<td>76</td>
</tr>
<tr>
<td><strong>Intracellular (IC) Regions</strong></td>
<td></td>
</tr>
<tr>
<td>1st IC Loop</td>
<td>89</td>
</tr>
<tr>
<td>2nd IC Loop</td>
<td>59</td>
</tr>
<tr>
<td>3rd IC Loop</td>
<td>78</td>
</tr>
</tbody>
</table>

**Table 3.** This table shows the sequence identity between all four receptors (DOR, MOR, KOR, and ORL-1) in human (hOR), rat (rOR), and mouse (mOR). Each receptor was aligned (using ClustalW) at the amino acid level for each organism and the sequence identity was based on identical residues among all four receptors. The sequences for each mammal can be found in the NCBI database using the following accession numbers: MMOR: NM_001039652, MORL1: NM_011012, MKOR: NM_011011, MDOR: NM_013622, HMOR: NM_001145282, HKOR: NM_000912, HDOR: NM_000911, HORL1: NM_182647, RDOR: NM_012617, RKOR: NM_017167, RMOR: NM_001038601, RORL1: NM_031569. Abbreviations are as follows: M = mouse, H = human, and R = rat.
3.2 Function and Distribution of Opioid Receptors

Opioid receptors couple to G proteins, GDP is replaced with GTP disassociating the alpha subunit which inhibits adenylyl cyclase and decreases cAMP within the cell. The beta-gamma subunit closes voltage-gated Ca\(^{2+}\) channels and activates inwardly rectifying K\(^{+}\) channels as shown in Fig 2 (reviewed in Kreek and LaForge 2007 and Dhawan et al., 1997). Activating inwardly rectifying K\(^{+}\) channel leads to a hyperpolarization, which will decrease the release of excitatory neurotransmitters (Endogenous Opioid Systems); this is referred to as antinociception. Although all three of the classic opioid receptors play a role in analgesia, each receptor also has several different functions that they exhibit based on their distribution within the central nervous system (CNS). Studies done in mammals including rat, mouse, and guinea pig revealed the distribution and function of each receptor.

The kappa opioid receptor is involved in nociception, diuresis, feeding, neuroendocrine secretions (Hansen and Morgan, 1984), dysphoria (Pfeiffer et al., 1986), miosis and respiratory depression (www.Opioids.com). KOR receptors are found within the inner layers of the cerebral cortex, substantia nigra, interpeduncular nucleus, nucleus accumbens, claustrum, and dorsal endopiriform nucleus (reviewed in Dhawan et al., 1996)

The delta opioid receptor plays a role in respiratory depression, gastro-intestinal motility, olfaction, cognitive function, analgesia, mood driven behavior, and motor integration. The olfactory bulb, neocortex, caudate putamen, and nucleus accumbens
show the highest density of DOR, while the thalamus, hypothalamus and brainstem illustrate moderate to low DOR densities (reviewed in Dhawan et al., 1996)

MOR is highly important in antinociception, blocking nociceptive responses to mechanical, thermal or chemical high intensity stimulations (Knapp et al., 1989). Stimulation of MOR has been shown to lead to depression of respiration, cardiovascular functions, intestinal transit, feeding, learning and memory, locomotor activity, thermoregulation, and immune functions. MOR are scattered throughout the neuraxis with the highest density found in the caudate putamen. MOR’s are also present in the neocortex, thalamus, nucleus accumbens, hippocampus, amygdale, periaqueductal gray, raphe nuclei and superficial layers of the dorsal horn. The lowest density of MOR’s are expressed in the hypothalamus, preoptic area, and globus pallidus (reviewed in Dhawan et al., 1996)

ORL-1 receptors are expressed in several different regions of the CNS, but especially within the brainstem, limbic areas, hypothalamus, and spinal cord (Mollereau et al., 1994). Because of its distribution within the CNS it is thought that ORL-1 may be involved in learning and memory, homeostasis, attention and emotions, and sensory perception. Once the endogenous ligand was discovered, it was shown that stimulation of ORL-1 caused an increased reactivity to pain (Meunier et al., 1995)

Because each of the opioid receptors are involved in a number of biological processes, it is imperative to study the phylogeny of these receptors in all of the major groups of vertebrates rather than making generalizations about structure/function relationships based only from studies on the mammalian opioid receptors.
Figure 3: Schematic diagram of opioid receptor signaling based on µ-opioid receptor function (Kreek and LaForge, 2007).
4. Opioid Receptors in Non-Mammalian Vertebrates

To better understand the past and present evolutionary path of the opioid/orphanin gene family it is important to have opioid receptor sequence information available over a broad taxonomic scale in lower vertebrates. The endogenous opioid ligands are well understood today because they have been fully cloned and characterized in several different organisms including lower vertebrates. This led to the discovery that opioid peptides had undergone several duplication events throughout their evolution (Dores et al., 1993). These duplications allow for mutations within each duplicated gene, which in turn permits different functionality within at least one of the gene duplicates (Danielson & Dores, 1999). This same concept is thought to be applied to the opioid receptor family. The opioid receptors however are not fully understood because extant representatives from the earliest class of vertebrates have yet to be fully cloned with the exception of zebra fish. To resolve this problem, attempts to fully clone the four opioid receptors from lamprey, horn shark, and white sturgeon are undertaken. This sequence information will give insight into the order in which each opioid receptor arose, help to identify ancient structural elements that may be essential for opioid receptor function, and later down the line will elaborate on the recent discoveries surrounding the differences in receptor binding affinity and signaling properties between non-mammalian vertebrates and mammalian vertebrates through evolution (Brasel et al., 2008).
Materials and Methods

1. Animals

White Sturgeon

Sexually immature white sturgeons, *Acipenser transmontanus* were obtained from Oregon State University (courtesy of Dr. Carl Shreck). Sturgeons were anesthetized by prolonged immersion in MS-222 (Sigma, St Louis, MO) and were sacrificed by decapitation. Their brains were rapidly removed and either placed in RNA Later (Ambion, Austin, TX) and stored at -20ºC or flash frozen in liquid nitrogen and stored at -80ºC until RNA isolation.

Horn shark

Horn sharks, *Heterodontus francisci* were collected at the Scripps Institute (San Diego, CA). The animals were anesthetized by lowering their body temperature to 10ºC. They were then decapitated; their brains were removed and immediately placed on dry ice. The brain samples were stored at -80ºC until RNA isolation.

Lamprey

Lampreys, *Petromyzon marinus*, were obtained from http://lampreyservices.com. Lampreys were anesthetized by prolonged immersion in MS-222 (Sigma, St Louis, MO) and were sacrificed by decapitation. Their brains were rapidly removed and either placed in RNA Later (Ambion, Austin, TX) and stored at -20ºC or flash frozen in liquid nitrogen and stored at -80ºC until RNA isolation.
2. Total RNA Extraction

The RNA extraction protocol used was developed by the W.M. Keck Foundation Biotechnology Microarray Resource Laboratory at Yale University. 50mg of previously extracted white sturgeon, Lamprey, and Horn shark brain were homogenized in 1mL of TRIzol reagent using a glass dounce. The samples were centrifuged for 90 seconds at room temperature to remove any non-homogenized debris and the supernatant was transferred to a fresh eppendorf tube, where they were incubated for five minutes at room temperature. 200µl of chloroform was added, the tube was shaken by hand vigorously for 15-30 seconds and then incubated for three minutes at room temperature. Centrifugation took place at 12,000g for 15 minutes at 2-8ºC. The RNA remained in the upper aqueous phase and was transferred to a fresh eppendorf tube. 500µl of Isopropyl alcohol was added, the tubes were vortexed for 10 seconds and incubated at room temperature for 10 minutes. Centrifugation took place at 12,000g for 10 minutes at 2-8ºC. The supernatant was removed by aspiration and the RNA pellet was washed once with 75% ethanol and vortexed. Centrifugation took place at 7,500g for five minutes at 2-8ºC. The supernatant was once again removed by aspiration and the RNA pellet was air-dried for 10 minutes. The RNA pellet was rehydrated in 22µl RNase-free water.

3. Chloroform Extraction

A chloroform clean-up was performed on the total RNA to extract the remaining phenol left from the TRIzol extraction. An equal amount of chloroform was added to the rehydrated samples, they were mixed by inverting five times and centrifuged at full speed for 5 minutes. The upper aqueous layer was transferred to a fresh eppendorf tube and
one-tenth of the total volume of NaOAc was added along with 3 times the total volume of 100% ethanol. Centrifugation took place at full speed for 10 minutes at 2-8°C. The supernatant was removed by aspiration and the pellet was washed with 70% ethanol (the volume used was half the total volume of the eppendorf tube) and centrifuged at room temperature for five minutes at full speed. The supernatant was removed once again by aspiration and the pellet was air-dried for ten minutes. The purified RNA pellet was rehydrated in 22µl of RNase-free water. 2µl of the purified total RNA product was analyzed using a Nanodrop ND1000. A 1.8 or higher 260/280 ratio was obtained each time. The 230/260 ratio was 2:1 or higher

4. Reverse Transcription

The total RNA products underwent reverse transcription using the Superscript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carslbad, CA). At least 1µg of total RNA was used in conjunction with the reverse transcription primer Anchor-dT17 (5’GAC TCG AGT CGG ATC CAT CGA T_{17}3’), which bound its seventeen thymine residues to the complementary poly-A tail of mRNA transcripts. The first strand of cDNA for all three species was then used in a polymerase chain reaction (PCR).

4.1 RT-PCR

A forward degenerate primer designed by Li et al. (1996): OpR-Fwd (5’GAARACIGCIACIAAYATHTA3’) and a degenerate reverse primer: JMDR (5’DATNTGDATNGNGTCCARC3’) were targeted to two highly conserved regions among all four opioid/orphanin receptors in zebrafish. The forward primer was targeted
to the sequence KTATNIY, found just outside and within the second transmembrane spanning region (TM2). The reverse primer was targeted to the sequence CWTPI(H,Q)I, found within the sixth transmembrane spanning region (TM6). This primer set was used on all three species and additional homologous primers were designed for the lamprey only (Figure 4).

Gene-specific forward and reverse primers were designed for each of the four receptors obtained from the lamprey genome. For the lamprey receptor 1A, a forward primer: LR.1A.Fwd (5’-AAAACCGCCACCAACAT-3’) and reverse primer: LR.1A.Rev (5’-CCAGCACACCACGAAG-3’) were targeted to the sequences KTATNI, within the first intracellular loop (ICL1), and AFVVCW within TM6 (Figure 5a). The 1B receptor forward primer: 1B.Fwd (5’-ACCGCTACCAACATCTAC-3’) was targeted to the sequence TATNIY, found between ICL1 and TM2, and the 1B reverse primer: LR.1B.Rev (5’-ACGTTGATGAGCTTGGC-3’) was targeted to the sequence AKL INV, found at the beginning of TM4 respectively (Figure 5b). A degenerate forward primer: LR.Degen.Fwd (5’-ARACSGCYACMAACAYBTAY-3’) and a gene-specific reverse primer: LR.2A.Rev (5’-GATAGACGAGAGCGAGCC-3’) were used to target sequences within the lamprey 2A receptor. The 2A forward primer was targeted to the sequence KTATNIY found between ICL1 and TM2 while the 2A reverse primer was targeted to the sequence WLLSSI found within TM4 (Figure 5c). Finally, the 2B forward primer: 2B.Fwd (5’-GAAGACGGCTACCAACACG-3’) was targeted to the sequence KTATNT, found between ICL1 and TM2, and the 2B reverse primer: LR.2B.Rev (5’-TTCTTGGCCGTGCCC-3’) was targeted to the sequence GTAKK, found at the end of TM4 (Figure 5d).
The PCR reagents were as follows: 2µl of each primer at 10µM each, 2µl of 10mM dNTP mix, 2µl of 10X PCR buffer (100mM Tris pH9.2, 750mM KCl, and 35mM MgCl$_2$), 9.8µl of dH$_2$O, 0.2µl of Taq polymerase at 5U/µl (Invitrogen, Carlsbad, CA) and 2µl of first strand cDNA template. The thermal profile allowed an initial pre-heat at 94°C for three minutes followed by 32 cycles, each cycle consisting of a forty-five second denaturing step at 94°C followed by an annealing step at 46.7°C for thirty seconds and an extension step at 72°C for one minute and a half. A final step at 72°C for ten minutes concluded the thermal profile.
**Figure 4:** Schematic diagram of the structure of opioid receptors based off of the MOR sequence from rat. The red bold circles indicate the sites where the forward and reverse degenerate primers were targeted for white sturgeon and horn shark. The forward degenerate primer designed by Li et al. was targeted to the amino acid sequence KTATNIY. The reverse degenerate primer was targeted to the amino acid sequence CWTPI(H,Q)I. The arrows represent the direction in which the primers were designed to work. Each transmembrane domain is clearly labeled as well as the extracellular space, intracellular space, and plasma membrane.
Figure 5a: Lamprey Opioid Receptor 1A

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Figure 5b: Lamprey Opioid Receptor 1B

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Figure 5c: Lamprey Opioid Receptor 2A

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121  CTCTGCAAGGTTGTGATGCCATCAGACTACATGCGTGGCGACGCTGCGCCATTGCGGCGCATC
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241  CGGACACGTACATGCGTGGCGACGCTGCGCCATTGCGGCGCATC
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301  GGCATTCCCATCATGATTATGGCGGCGACGCTGCGGCGCATC
101  -G--V--P--I--M--I--M--G--T--I--K--P--D--Q--I--
```

Figure 5d: Lamprey Opioid Receptor 2B

```
1  ATGAAGACGGCTACCAACACGTACATCTTTCACTTGCGCGACGACACATGCTGACG

61  AGCACGCTGGCCGTCATGCTGCGATGCGACGCTGCGCCATTGCGGCGCATC

121  ATCTGCAAGGTTGTGATGCCATCAGACTACATGCTGCGTGGCGACGCTGCGCCATTGCGGCGCATC
41  -I--C--K--V--V--M--S--I--D--Y--Y--N--M--F--T--S--I--F--T--L--

181  ACGATCATGAGGTCGATGCTATCGTACATGCGTGGCGACGCTGCGCCATTGCGGCGCATC

241  CGGACACGTACATGCGTGGCGACGCTGCGCCATTGCGGCGCATC
81  -R--T--P--S--K--A--K--L--I--N--I--W--I--L--S--S--A--I--

301  GGCATTCCCATCATGATTATGGCGGCGACGCTGCGGCGCATC
101  -G--I--P--I--M--I--M--G--T--I--K--P--D--Q--I--
```

Figure 5 a-d: Putative lamprey opioid receptors sequences at the nucleotide and amino acid levels. The gray highlighted sequence represents where each primer was targeted and the arrows represent which direction the primers were designed to work. The lamprey sequences for 1A, 1B, 2A, and 2B can be found in the Ensembl Lamprey Genome under the contigs: 51033.1 900-2052, 55613.1 4454-5237, 28623.2 980-1325, 7520.3 1519-1861 respectively.
5. Gel Electrophoresis

The PCR products were analyzed using a 1% agarose gel made by combining 1g of agarose (ISC BioExpress, Kaysville, UT) with 100mL of 1X TAE buffer. The 1X TAE buffer was composed of 4.48g Tris, 1.142mL Acetic Acid, 0.5M EDTA (ph 8), and 1L dH₂O. 10µl of 1mg/mL ethidium bromide (Sigma-Aldrich, St. Louis, MO) was added to the 1% agarose gel mixture. The PCR products were loaded into the gel with a 6X loading dye (Promega, Madison, WI) and a low range 100bp ladder (Fisher Scientific, Pittsburg, PA). The gel ran at 100-110V for 20-30 minutes and was then placed into a molecular imaging gel doc (Biorad, Hercules, CA) for visualization. The RT-PCR yielded a 600bp target band that was excised using Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI).

6. Ligation and Transformation

The cleaned PCR product was ligated into the pGEM-T vector (Figure 6) at a 3:1 insert:vector ratio according to pGEM-T and pGEM-T Easy Vector Systems (Promega, Madison, WI). The vector was then inserted into Z-competent *E.coli* DH5α cells (Zymo Research, Orange, CA) for transformation following the manufacturer’s recommendations. The bacteria containing the vector were grown on LB-Agar-Ampicillin plates overnight. The LB-Agar-Amp plates were created by combining 10 g Tryptone, 5g yeast extract, 5g NaCl, 12g agar, and 1L dH₂O. The transformants were selected by blue-white color screening. The white colonies were screened for the correct size insert using rapid cycle PCR (Wittwer et al, 1994). A small portion of the colony was picked off with a sterile pipette tip and dissolved in 10µl of water. 1µl of the dissolved
bacterial colony was used in a rapid cycle PCR as the DNA template. Gene specific primers, OpR-FWD and JM Degen REV, were used to ensure that the correct product was present within the vector. The PCR reagents included 1µl of each primer at 10µM, 1µl of 10mM dNTP mix, 2µl of 10X Mg$^{2+}$ buffer (500mM Tris pH 8.3, 2.5mg/mL BSA, and 30mM MgCl$_2$), 0.1µl of Taq polymerase (Invitrogen, Carlsbad, CA) and 3.9µl of dH$_2$O. The thermal profile consisted of an initial denaturing step at 94ºC for two minutes followed by an additional 32 cycles, each cycle consisted of an annealing step at 47ºC for 15 seconds and an extension step at 72ºC. The rapid cycle PCR products were then electrophoresed on an agarose gel as described above.

7. Colony Touch

A colony touch protocol developed by Hofmann and Brian 1991 was used to identify the plasmid DNA. The colonies containing the correct insert, according to the rapid cycle PCR, were picked off using a sterile pipette tip and dissolved in 100µL of T.E.(10mM Tris, 0.1mM EDTA, pH 8). They were heated for 10 minutes at 100ºC and 2µl was used as the DNA template in a 25µl PCR reaction. The PCR reagents were as follows: 2µl of each vector primer (SP6 and T7) at 10µM each, 2µl of 10mM dNTP mix, 2µl of 10X PCR buffer (100mM Tris pH9.2, 750mM KCl, and 35mM MgCl$_2$), 14.8µl of dH$_2$O, 1U of Taq polymerase at 5U/µl (Invitrogen, Carlsbad, CA). The thermal profile allowed an initial pre-heat at 94ºC for three minutes followed by 32 cycles, each cycle consisting of a forty-five second denaturing step at 94ºC followed by an annealing step at 55ºC for thirty seconds and an extension step at 72ºC for one minute and a half. A final step at 72ºC for ten minutes concluded the thermal profile. 5µl of the PCR product was
electrophoresed on an agarose gel as described above. The products containing the
correct sized insert (size of the cloned insert plus the size of the multiple cloning region
within the vector) were prepared for sequencing as follows: 5U of Exonuclease I (5U/µl,
USB, Cleveland, OH) and 0.5U of shrimp alkaline phosphatase (1U/µl, USB, Cleveland,
OH) was added to the remaining 20µl PCR product and a 37°C incubation followed. The
enzymes were heat-inactivated by a 15 minute incubation at 80°C.
Figure 6: pGEM-T vector circle map and sequence reference points (Promega, Madison, WY). The gray highlighted section shows the T7 RNA polymerase transcription initiation, multiple cloning region, and SP6 RNA polymerase initiation site.
8. Sequencing

The purified PCR products were labeled for sequencing using the CEQ-DTCS Quickstart Kit (Beckman-Coulter, Fullerton, CA) following the manufacturer’s recommendations and sequenced on the CEQ 8000 XL DNA Analysis System (Beckman-Coulter, Fullerton, CA). The sequence data chromatograms were analyzed using Sequencher 4.9 (Gene Codes, Ann Arbor, MI). The sequences were submitted to NCBI’s BLAST search engine and compared to known sequences.

9. Primer Design

White Sturgeon and Horn Shark

All four zebrafish opioid receptors were aligned at the amino acid level using the multiple alignment program Clustal W2. Opioid/orphanin sequences from human, chicken, xenopus tropicalus, and zebrafish were also aligned at the amino acid level for each individual receptor using ClustalW2. Based on these alignments, highly conserved regions within the opioid/orphanin receptors were chosen as targets for primer design (Figure 4). Each primer designed was then analyzed for hairpin loops, 3’ complementarities, and self-annealing sites using the Oligonucleotide Properties Calculator (Northwestern University Medical School, Chicago, IL).

Lamprey

The Ensembl Genome Browser was utilized to probe the Lamprey genome for possible opioid/orphanin receptor sequences. Using the sea lamprey genus and species, *Petromyzon marinus*, known opioid/orphanin receptor sequences from zebrafish were use
to screen the entire lamprey genome at the amino acid level using the BLASTN option. Once the results were retrieved, the “graphical view” revealed the alignment and location of the closest match. Both the nucleotide and amino acid sequences were displayed and through a process of elimination, based on conserved transmembrane domains within opioid/orphanin sequences, four lamprey receptors sequences assigned the names 1A, 1B, 2A, and 2B were retrieved. Using those nucleotide sequences forward and reverse primers were designed for each of the four different lamprey receptor sequences (see Fig 5 a-d). The primer design was analyzed using Oligonucleotide Properties Calculator as above.

10. Phylogenetic Analyses

Alignments performed by ClustalW were refined manually following the procedures outlined in Dores et al (1996). Phylogenetic analyses were aligned and analyzed using the exhaustive search mode of the maximum parsimony algorithm (PAUP 4.1). Lamprey prosomatostatin (Lamprey Genome Project; Contig 25590.1 314-1366) was used as the outgroup.
Results

A. RT-PCR

The first round of RT-PCR yielded a fragment from lamprey, horn shark and white sturgeon that were approximately 600 base pairs (bp) in length. The fragment was amplified using the degenerate forward primer (OpR-Fwd) designed by Li et al. in (1996) and the JMDR reverse primer. OpR-Fwd was targeted to the sequence KTATNIY, found just outside and within the second transmembrane spanning region (TM2) and JMDR was targeted to the sequence CWTP(H,Q)I, found within the sixth transmembrane spanning region(TM6).

Using the Primer Design procedure for Lamprey outlined in the Materials and Methods section, four separate lamprey receptor fragments were also obtained. The lamprey receptor 1A yielded a 573bp fragment, 1B yielded a 266bp fragment, 2A yielded a 291bp fragment, and 2B yielded a 333bp fragment.

After the fragments were isolated a total of ten bacterial colonies from white sturgeon, ten bacterial colonies from horn shark and thirty bacterial colonies from lamprey were screened, sequenced, and submitted the National Center for Biotechnology Information (NCBI) database using the blastn (nucleotide blast) option as described in Materials and Methods.
The clones were compared to a collection of sequences submitted to the NCBI database for identification. The white sturgeon clones revealed five kappa-like sequences and five ORL-like sequences. Of the ten horn shark clones, all were identified as delta-like sequences. The lamprey clones revealed fifteen delta-like sequences, ten delta variants, and five mu-like sequences.

The raw sequence data were then used to create a consensus sequence for each receptor. The chromatograms for each sequence in every receptor were aligned and analyzed in Sequencher. Each base corresponded to a certain peak in the chromatograms; therefore, every base that differed from the others was either changed or deleted according to the strength of the peaks that corresponded to the most frequently occurring base at that point in the sequence. Through this process a consensus sequence for each receptor in every organism emerged.

**B. Lamprey Receptors**

The fragment (lamprey DG) obtained from lamprey using the degenerate forward primer OPR-Fwd and the degenerate reverse primer JMDR (for reference see primer design in Materials and Methods section) spans from the second transmembrane domain to the sixth transmembrane domain. The deduced amino acid sequence is 204 residues in length and includes the end of the first intracellular loop, the entire second and third intracellular loops, and the entire first and second extracellular loops (Figure 7). The cloned lamprey fragment 2A is a smaller portion of this cloned fragment and from here forward will be referred to with the lamprey DG.
Figure 7 Lamprey DG: Deduced amino acid and nucleotide sequence of the putative lamprey DOR fragment from the RT-PCR using the forward degenerate primer designed by Li et al. (1996) and the reverse primer JM Degen Rev. Transmembrane domains are highlighted in gray with the TM number above and the sequence to which the primers were targeted are underlined.
The lamprey 1A fragment obtained using homologous primers targeted to the first intracellular loop and the sixth transmembrane region (for reference see primer design in Materials and Methods section) has a deduced amino acid sequence of 191 residues, which includes the end of the first intracellular loop, the entire second and third intracellular loops, and the entire first and second extracellular loops (Figure 8).

The fragment for lamprey 1B was retrieved using homologous primers targeted to the second transmembrane domain and the fourth transmembrane domain (for reference see primer design in Materials and Methods section). It has a deduced amino acid sequence of 88 residues which includes transmembrane domains 1-4, the end of the first intracellular loop, the entire second intracellular loop, and the entire first extracellular loop (Figure 9).

The lamprey 2A fragment was obtained using a degenerate forward primer targeted to the end of the first intracellular loop and the beginning of the second transmembrane domain and a homologous reverse primer targeted to the fourth transmembrane domain (for reference see primer design in Materials and Methods section). The deduced amino acid sequence is ninety-seven residues in length turned out to be an identical smaller portion of the lamprey DG fragment. It represents amino acid residues 2-97 of lamprey DG (Figure 7) and from this point forward will be combined with the lamprey DG fragment.
Figure 8 Lamprey 1A: Deduced amino acid and nucleotide sequence of the putative lamprey DOR fragment from the RT-PCR using a homologous forward and reverse designed as mentioned above in the Materials and Methods section. Transmembrane domains are highlighted in gray with the TM number above and the sequence that the forward primer was targeted to is underlined.
Figure 9 Lamprey 1B: Deduced amino acid and nucleotide sequence of the putative lamprey MOR fragment from the RT-PCR using a homologous forward and reverse designed as mentioned above in the Materials and Methods section. Transmembrane domains are highlighted in gray with the TM number above and the sequence that the primers were targeted to are underlined.
Finally, the lamprey 2B fragment was retrieved using a homologous forward primer targeted between the end of the first intracellular loop and the second transmembrane region and a homologous reverse primer targeted to the end of the fourth transmembrane region (for reference see primer design in Materials and Methods section). It has a deduced amino acid sequence of 111 residues that includes transmembrane domains 1-4, part of the first intracellular loop, the entire second intracellular loop, the entire first extracellular loop, and the beginning of the second extracellular loop (Figure 10).
**Figure 10 Lamprey 2B:** Deduced amino acid and nucleotide sequence of the putative lamprey DOR variant fragment from the RT-PCR using a homologous forward and reverse designed as mentioned above in the Materials and Methods section. Transmembrane domains are highlighted in gray with the TM number above and the sequence that the primers were targeted to are underlined.
C. Horn Shark Receptors

The horn shark fragment obtained using the degenerate OPR-Fwd primer and JMDR reverse primer (for reference see primer design in Materials and Methods section) spans from the second transmembrane domain to the sixth transmembrane domain. The deduced amino acid sequence is 200 residues in length and includes the end of the first intracellular loop, the entire second and third intracellular loops, and the entire first and second extracellular loops (Figure 11).

D. White Sturgeon Receptors

The fragments obtained from the white sturgeon using the degenerate OPR-Fwd primer and degenerate JMDR reverse primer span from the second transmembrane domain to the sixth transmembrane domain with a deduced amino acid sequence of 199 residues for the kappa-like sequences and 201 residues for the ORL-like sequences. The fragments contain part of the first intracellular loop, the entire second and third intracellular loops, and the entire first and second extracellular loops. The ORL-like fragment is shown in Figure 12 and the kappa-like receptor is shown in Figure 13.
Figure 11 HS DOR: Deduced amino acid and nucleotide sequence of the putative horn shark DOR fragment from the RT-PCR using the forward degenerate primer designed by Li et al. (1996) and the reverse primer JM Degen Rev. Transmembrane domains are highlighted in gray with the TM number above and the sequence that the primers were targeted to are underlined.
Figure 12 WS ORL: Deduced amino acid and nucleotide sequence of the putative white sturgeon ORL fragment from the RT-PCR using the forward degenerate primer designed by Li et al. (1996) and the reverse primer JMDR. Transmembrane domains are highlighted in gray with the TM number above and the sequence that the primers were targeted to are underlined.
Figure 13 WS KOR: Deduced amino acid and nucleotide sequence of the putative white sturgeon Kappa-like fragment from the RT-PCR using the forward degenerate primer designed by Li et al. (1996) and the reverse primer JM Degen Rev. Transmembrane domains are highlighted in gray with the TM number above and the sequence that the primers were targeted to are underlined.
E. Vertebrate Amino Acid Alignments

Each partial receptor sequence was aligned with other known vertebrate sequences to provide further support to the BLAST results. A representative from each vertebrate group was used to provide a complete picture of the opioid/orphanin receptor vertebrate evolution. Humans were used for the mammalian group, chicken was used for the avian species, either rough-skinned newt (Taricha granulose) or Xenopus tropicalis was used for the amphibian group, and zebrafish was used for the fish group. The animals cloned in this study extend the fish species to include the oldest members of the vertebrate class, with lamprey representing jawless fish, horn shark representing cartilaginous fish, and white sturgeon representing ray-finned fish. Comparing the amino acid sequence identities over a broad taxonomic range further establishes the placement and evolutionary relationship of each receptor clone. It provides another visual understanding of how closely opioid/orphanin receptors are related.

The lamprey fragments all showed a close relationship according to the preliminary BLAST results, therefore all of the lamprey clones were aligned together with other vertebrate DOR’s (Figure 18). However, because the LR DG fragment, based on the primary sequence, was shown to be more delta-like with the BLAST search and more mu-like with the phylogenetic analysis, it was aligned with both DOR and MOR vertebrates to reveal which receptor it shared the most sequence identity. The LR DG receptor shows the highest sequence identity with DOR’s in the intracellular loops with ranges from 90% in newt and zebrafish to 94% in chicken. The zebrafish DOR shows the lowest sequence identity with the LR DG fragment at only 72%. The ZDOR transmembrane regions, which are normally highly conserved among opioid receptors, share only 71% sequence identity with the LR DG fragment (Table 3). When aligned
with vertebrate MOR’s (Figure 19), the LR DG fragment shares 83% sequence identity with the ZMOR within the transmembrane domains and an overall sequence identity of 76% (Table 3). Among the different vertebrates included in the alignments, zebrafish should show the highest sequence identity with the LR DG fragment because they both belong to the vertebrate class of fish. Taking this into consideration, along with the phylogenetic analysis, it seems likely that the LR DG fragment is more closely related to MOR.

The lamprey 1A fragment shows the highest sequence identities within the transmembrane domains and the intracellular loops (Figure 18). Sequence identities within the intracellular loops ranged from 86% in chicken to 92% in newt and zebrafish. The newt and zebrafish also showed the highest amino acid identity within the transmembrane domains at 72%. Interestingly, the extracellular loops seem to increase in sequence identity as the vertebrates evolve with the lowest sequence identity present in zebrafish at only 49%. In general, the LR 1A fragment shows 71%-73% overall sequence identity with vertebrate DOR’s (Table 4).

The lamprey 1B and 2B fragments both show very high sequence identity with vertebrate DOR’s due in part to the small portion of amino acid residues involved in the alignment (Figure 18). LR 1B and 2B were only 88 amino acids and 111 amino acid residues in length, respectively. Both fragments show the highest sequence identities within the transmembrane domains and intracellular loops with identities ranging from 85-96%. Even within the extracellular loops, LR 1B shows 61% sequence identity and LR 2B shows 67% sequence identity. Both fragments show an overall 82-85% sequence identity with other vertebrate DOR’s (Table 5).
**Figure 14 DOR vertebrate alignment:** Each animal was aligned using ClustalW and only the conserved regions for *all* five groups are shaded in gray. (-) represent gaps inserted where no base is present. The vertebrate sequences for human, chicken, rough-skinned newt, and zebrafish can be found in the NCBI database using the accession numbers: NM_000911.3, XM_427506, AY751785, and NM_212755 respectively.
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### LR DG DOR Amino Acid % Identities

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**Table 3 LR DG Amino Acid % Identity:** This table shows the amino acid % identities of the intracellular loops (ICL), extracellular loops (ECL), and transmembrane domains (TM) for only the partial (204 residues) amino acid fragment cloned. Amino acid % identities are present for human, chicken, Xenopus tropicalis (X.trop), and zebrafish. Percent identities include the gaps inserted during the multiple sequence alignment performed by ClustalW.
**Figure 15 MOR vertebrate alignment:** Each animal was aligned using ClustalW and only the conserved regions for *all* five groups are shaded in gray. (-) represent gaps inserted where no base is present. The vertebrate sequences for human, chicken, Xenopus tropicalis, and zebrafish can be found in the NCBI database using the accession numbers/protein ID’s: NM_000914.3, ENSGALP00000022114, ENSXETP00000018406, and NM_131707 respectively.
The horn shark (HS) fragment shows a great deal of conservation with the DOR’s vertebrates (Figure 18). Although the phylogenetic analysis could not determine if it was more delta-like or mu-like, the vertebrate alignment shows that it shares a high amount of sequence identity with all of the vertebrates. Within the most conserved region, the intracellular loops, the HS fragment shares 94% sequence identity with both newt and zebrafish. The transmembrane regions show sequence identities ranging from 77-90% and even the extracellular loops show sequence identities as high as 73% in human and chicken. Overall sequence identities ranging from 80-86% show that the horn shark fragment is most similar to the DOR (Table 6).

The sturgeon ORL-fragment turned out to be less conserved than the KOR fragment with an overall sequence identity ranging from 73-80% within the partially cloned 201 amino acid residue region (Figure 20). The extracellular loops are poorly conserved with as low as 43% sequence identity in newt, 52% identity in human and chicken, and 67% in zebrafish. The transmembrane domains and intracellular loops are the most highly conserved with amino acid identities ranging from 74%-94%. Overall, the zebrafish again shares the most sequence identity with white sturgeon ORL fragment at 80% while the human shares the lowest sequence identity at 73% (Table 7).
**LR 1A DOR Amino Acid % Identities**

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**Table 4 LR 1A Amino Acid % Identity:** This table shows the amino acid % identities of the intracellular loops (ICL), extracellular loops (ECL), and transmembrane domains (TM) for only the partial (191 residues) amino acid fragment cloned. Amino acid % identities are present for human, chicken, Xenopus tropicalis (X.trop), and zebrafish. Percent identities include the gaps inserted during the multiple sequence alignment performed by ClustalW.
Table 5 LR 1B & 2B Amino Acid % Identity: This table shows the amino acid % identities of the intracellular loops (ICL), extracellular loops (ECL), and transmembrane domains (TM) for only the partial (88 residues for 1B and 111 residues for 2B) amino acid fragment cloned. Amino acid % identities are present for human, chicken, xenopus tropicalis (x.trop), and zebrafish. Percent identities include the gaps inserted during the multiple sequence alignment performed by ClustalW.
**Figure 16 ORL vertebrate alignment:** Each animal was aligned using ClustalW and only the conserved regions for all five groups are shaded in gray. (-) represent gaps inserted where no base is present. The vertebrate sequences for human, chicken, rough-skinned newt, and zebrafish can be found in the NCBI database using the accession numbers: U30185, XM_417424, AY728087.1, and AY148348 respectively.
### Table 6 HS DOR Amino Acid % Identities

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<td>86%</td>
<td>82%</td>
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**Table 6 HS DOR Amino Acid % Identity:** This table shows the amino acid % identities of the intracellular loops (ICL), extracellular loops (ECL), and transmembrane domains (TM) for only the partial (202 residues) amino acid fragment cloned. Amino acid % identities are present for human, chicken, Xenopus tropicalis (X.trop), and zebrafish. Percent identities include the gaps inserted during the multiple sequence alignment performed by ClustalW.
Figure 17 KOR vertebrate alignment: Each animal was aligned using ClustalW and only the conserved regions for all five groups are shaded in gray. (-) represent gaps inserted where no base is present. The vertebrate sequences for human, chicken, xenopus tropicalis, and zebrafish can be found in the NCBI database using the accession numbers/protein ID’s: NM_000912.3, XM_426087, ENSXETP00000017733, and NM_182886 respectively.
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<table>
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<td><strong>Overall</strong></td>
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<td><strong>KOR Amino Acid % Identities</strong></td>
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**Table 7 WS ORL/KOR Amino Acid % Identity:** This table shows the amino acid % identities of the intracellular loops (ICL), extracellular loops (ECL), and transmembrane domains (TM) for only the partial (201 residues for ORL and 199 residues for KOR) amino acid fragment cloned. Amino acid % identities are present for human, chicken, Xenopus tropicalis (X.trop), and zebrafish. Percent identities include the gaps inserted during the multiple sequence alignment performed by ClustalW.
KOR receptors appear to be highly conserved among vertebrates (Figure 21) showing 81-89% overall sequence identity within the partially cloned sturgeon region (202 amino acid residues including gaps). The intracellular loops provide the highest amino acid identity ranging from 94% with zebrafish to 96% with chicken and human. The extracellular loops are the most divergent with sequence identities as low as 51% with human to sequence identities as high as 83% with zebrafish. The transmembrane domains are also highly conserved among all vertebrates with 82-89% sequence identity. As expected, zebrafish shows the highest overall sequence identity with white sturgeon because of its close evolutionary position to sturgeon with 89% overall identity (Table 7). Human, chicken, and Xenopus tropicalis all share approximately the same overall sequence identity with sturgeon.

Although the vertebrate alignments, amino acid percent identity tables, and the BLAST searches provided substantial evidence for the identity of each cloned fragment, phylogenetic analyses were performed to add further support and confirmation of these results.

F. Phylogenetic Analysis

The cloned fragments were further compared using an exhaustive search mode of the maximum parsimony algorithm (PAUP 4.1). Each clone was analyzed with all four zebrafish receptors to elucidate their evolutionary order and further confirm which receptor, from the species presented in Section E, each fragment is most closely aligned. Lamprey somatostatin (LRSS) was used as the outgroup because this receptor is also a member of the Rhodopsin family of G-Protein coupled receptors and Blast P indicates
that somatostatin receptors have the highest sequence identity to opioid receptors. Somatostatin receptors are encoded on intron-less genes and were used by Li et al. (1996) as the group for their seminal study on opioid-related receptors in the hagfish brain. To test the robustness of each phylogenetic tree, a bootstrap analysis was performed using 1000 replicates and a confidence level for each clade that was present within each tree was assigned. Bootstrapping is a technique that resamples the original data set a number of times to see if the outcome (most parsimonious tree) remains the same. The bootstrap value represents how many times the resampled data support the original most parsimonious tree (Graur and Li, 1999).

The phylogenetic analysis showed that the lamprey DG (LR DG) fragment was most closely related to zebrafish MOR (ZMOR) with a confidence level of only 50%. The tree shows that ZMOR and LR DG are sister taxa that are connected to the Zebrafish DOR (ZDOR) with 93% confidence level. This means that 93% of the trees created by resampling the data 1000 times through bootstrapping, support this clade. Zebrafish KOR (ZKOR) and ORL (ZORL) do not share a clade with the LR DG fragment but are related to one another. Overall the phylogenetic study suggests that LR DG is most closely related to both ZMOR and ZDOR but not to ZORL and ZKOR (Figure 14a).
Figure 18 a-b: Phylogenetic analysis of lamprey DG and lamprey 1A based only on the partial fragment size (204 and 191 amino acid residues respectively). Each lamprey species was compared to all four zebrafish opioid/orphanin receptors using somatostatin as an outgroup. Each phylogenetic tree was created using the phylogenetic analysis using parsimony (PAUP). A bootstrap value for each clade within each tree was assigned based on 1000 replicates.
The tree produced for the lamprey 1A (LR 1A) fragment shows that its most closely related to ZDOR with a 76% confidence level. LR 1A and DOR are sister taxa that are connected to, but separate from, ZMOR with a 96% confidence level. ZKOR is equally related to ZDOR, LR 1A, and ZMORT with a 97% confidence level. ZORL is the most divergent from 1A and the closest to ZKOR (Figure 14b).

The phylogenetic analysis for the lamprey 1B (LR 1B) fragment presents a simple relationship. The LR 1B fragment cannot be distinguished as being more delta or mu-like possibly due to its smaller fragment size (88 amino acid residues). Therefore, all three receptors share a clade as sister taxa with an 80% confidence level. They are connected to the ZKOR with a 68% confidence level and again are most divergent from the ZORL. The LR 1B fragment could be considered as either a delta or mu variant (Figure 15a).

The lamprey 2B (LR 2B) fragment does not show a clear relationship to either ZDOR or ZMOR. The tree shows that ZDOR and ZMOR are sister taxa with an 86% confidence level. They do share a clade with 2B showing that they are related but that they branched off separately with an 86% confidence level. The LR 2B fragment also shares a clade with the ZKOR fragment expressing a 76% confidence level. ZORL is again the most divergent from ZDOR, ZMOR, and LR 2B (Figure 15b).

The horn shark DOR (HS DOR) fragment shows a similar relationship as the LR 1B fragment. The relationship between delta and mu is not clear and therefore, the HS fragment is a sister taxa of both receptors sharing a clade with 97% confidence. Although the initial nucleotide BLAST showed that the sequence was more delta-like, the phylogenetic analysis suggests that it may be a delta or mu variant (Figure 16).
Figure 19 a-b: Phylogenetic analysis of lamprey 1B and lamprey 2B based only on the partial fragment size (88 and 111 amino acid residues respectively). Each lamprey species was compared to all four zebrafish opioid/orphanin receptors using somatostatin as an outgroup. Each phylogenetic tree was created using the phylogenetic analysis using parsimony (PAUP). A bootstrap value for each clade within each tree was assigned based on 1000 replicates.
Figure 20: Phylogenetic analysis of the horn shark fragment based only on the partial fragment size (200 amino acid residues respectively). The horn shark was compared to all four zebrafish opioid/orphanin receptors using somatostatin as the outgroup. Each phylogenetic tree was created using the phylogenetic analysis using parsimony (PAUP). A bootstrap value for each clade within each tree was assigned based on 1000 replicates.
The white sturgeon ORL (WS ORL) fragment is the only receptor in the tree that is connected to the ZORL indicating that WS ORL is most similar to ZORL. The WS ORL is also not connected to ZDOR or ZMOR, but it is connected to ZKOR with a 69% confidence level. The lack of relatedness between ORL with any of the other receptors represents the evolutionary divergence that is present between ORL with DOR and MOR. As expected, the ZDOR and ZMOR are sister taxa sharing a clade with 98% confidence (Figure 17a).

The phylogenetic tree created for white sturgeon KOR (WS KOR) starts to unveil the classic relationship between the opioid/orphanin receptors. The ZDOR and ZMOR are sister taxa sharing a clade with 80% confidence. The WS KOR fragment and the ZKOR also represent sister taxa with a relatively high confidence level of 95%. The tree confirms with 100% confidence that each clade of sister taxa branched in this exact order (Figure 17b).
Figure 21 a-b: Phylogenetic analysis of sturgeon ORL and KOR based only on the partial fragment size (201 and 199 amino acid residues respectively). Each sturgeon species was compared to all four zebrafish opioid/orphanin receptors using somatostatin as an outgroup. Each phylogenetic tree was created using the phylogenetic analysis using parsimony (PAUP). A bootstrap value for each clade within each tree was assigned based on 1000 replicates.
To complete the evolutionary scheme between all vertebrates, each animal was further compared to expose their evolutionary relationship using a heuristic search mode of the maximum parsimony algorithm (PAUP 4.1). An exhaustive search with bootstrap values was too rigorous for the algorithm’s capabilities. The evolutionary relationship between opioid/orphanin receptors is clearly laid out in the final phylogenetic tree. It plainly shows that ORL’s are the most divergent from every other receptor except KOR’s. DOR’s and MOR’s are most closely related to each other and only distantly related to ORL’s through the KOR’s.

The LR 2B fragment is connected to the clade that splits MOR’s and DOR’s meaning that it is indistinguishable as either but is directly related to both. The LR DG and LR 1B fragments are most similar to each other and most closely related to the MOR’s, which presented a great evolutionary stair step: lamprey → zebrafish → xenopus tropicalis → chicken → human. The LR 1A fragment and the HS fragment are most similar to the DOR’s which, for the most part, also provide a classic evolutionary pathway: lamprey → zebrafish → horn shark → xenopus tropicalis → human → chicken. The glue that holds the opioid/orphanin receptor family together seems to be the KOR’s because they are directed related to both the DOR/MOR group and to ORL’s. The WS KOR is most similar to the ZKOR, and the group follows the pattern: white sturgeon → zebrafish → xenopus tropicalis → human → chicken. The ORL’s are a little more confusing showing the WS ORL being directly connected to the KOR cluster and the ZORL. Overall, it clear that ORL is the most distant receptor directly related to only KOR, and KOR is related to both MOR and DOR (Figure 22).
**Figure 22 Vertebrate Phylogenetic Tree:** Phylogenetic analysis of all four opioid/orphanin receptors using somatostatin (LRSS) as an outgroup. Each phylogenetic tree was created by a heuristic search using the phylogenetic analysis using parsimony (PAUP). Each species is abbreviated as follows: Human = H, Chicken = C, Newt = N, Xenopus tropicalis = X, Zebrafish = Z, White Sturgeon = WS, Horn shark = HS, and Lamprey = LR.
Discussion

Because the opioid/orphanin gene family are members of the G protein-coupled super family they are highly structurally conserved. They all share common structural characteristics that serve specific functions. All members of the G protein-coupled receptor superfamily share an extracellular N-terminal, intracellular C-terminal and seven transmembrane domains. Within the opioid receptor family, there are also other conserved regions that seem to elucidate receptor functionality and ligand binding. In mammalian opioid receptors, the highest conservation lies within the transmembrane domains and intracellular loops accounting for 42-76% (excluding TM4) and 59-89% receptor identity, respectively (Tables 3-7). This vast conservation among the opioid/orphanin gene family is believed to be a result of whole genome duplication. The most divergences among mammalian opioid receptors exist within the extracellular loops, which has led researchers to propose that these extracellular loops are significant in ligand selectivity. The zebrafish has been the only fish available with full sequence identity for each of the four receptors. Therefore, by cloning extant representatives from early vertebrates such as the lamprey, horn shark, and white sturgeon, it becomes more evident when these genome duplications took place and offers more insight about the ancient structural similarities that these receptor share.
1. Ligand/Receptor Binding

The vertebrate protein alignments revealed that all of the cloned receptor fragments demonstrate 71-91% sequence identity among transmembrane domains from jawless fish to mammals (Tables 3-7). This high conservation between transmembrane domains in opioid/orphanin receptors has been shown to be responsible for ligand binding. Opioid/orphanin receptors have seven transmembrane domains that are believed to be tightly associated within the membrane and folded into a helical bundle with an internal aromatic binding pocket. Using three-dimensional computer modeling of the DOR, the opioid binding pocket was shown to span across TM III through TM VII. Critical residues within this aromatic pocket include tyrosine, phenylalanine, and tryptophan residues. Site-directed mutagenesis of these critical aromatic residues has been shown to dramatically affect ligand binding (Befort et al., 1996). The residues within the aromatic pocket are highlighted in Figure 23. Reviewing the transmembrane regions in the cloned receptor fragments revealed that the most divergent transmembrane region within all of the vertebrate alignments was undoubtedly TM IV with only seven conserved regions. The tryptophan residue found within this transmembrane region is one of the critical aromatic residues found within the opioid binding pocket. It is conserved not only in every cloned receptor fragment, but also in all four vertebrates used in the alignments and all four receptors. In fact, all of the cloned receptor fragments, although presented over three different vertebrate classes, display conservation among these critical aromatic residues suggesting that they are indeed very significant in opioid receptor binding (Figures 18-21).
**Figure 23**: Schematic depiction of the opioid/orphanin receptor structure. Seven transmembrane spanning domains with highlighted amino acids representing the critical amino acid residues which make up the opioid binding pocket (Befort et al., 1996).
2. Ligand/Receptor Selectivity

The most divergent regions within the opioid receptors lie within the extracellular loops. Chimeric µ/κ and κ/δ receptors have revealed which extracellular loops are important within each receptor for ligand selectivity. It has been shown that ECL2 and the top of TM4 are involved in kappa selectivity, ECL3 is involved with delta selectivity, and ECL2 and ECL3 are responsible for mu selectivity (Jordan et al., 1998). The second extracellular loop is also important in ORL selectivity due to its highly negative charge, a characteristic it shares with the kappa extracellular loop. The KOR vertebrate alignment highlights only twelve conserved amino acid residues within ECL2 giving it the highest sequence identity between all of the ECL’s in all of the receptors at 41% (Figure 24a). The alignment for MOR shows that the second and third extracellular loops are highly divergent among vertebrates including the newly cloned LR DG fragment. Only nine residues remain conserved within ECL2 resulting in 31% sequence identity, and seven residues for ECL3 resulting in 27% sequence identity (Figure 24b). The third extracellular loop is significant for DOR, and the alignment shows that only four amino acid residues are conserved within ECL3 exhibiting only 23% sequence identity (Figure 24c). The second extracellular loop in ORL shows the highest amount of divergence with only four amino acid residues conserved and only 14% sequence identity (Figure 24d). Because these regions are so divergent, the conserved residues must play an important role in opioid/orphanin selectivity. The disulfide bridge between EC1 and EC2 is also conserved within all the receptors cloned from lamprey, horn shark, and white sturgeon. This further confirms the importance of these residues in stabilizing the functional receptor protein.
In 1996 Meng and colleagues took full advantage of the divergence within the extracellular loops in a quest to discover which amino acid residues play a role in ligand selectivity. They used the Orphanin FQ (ORL-1) receptor due to its lack of binding capabilities with any other classic opioid ligand. Their goal was to give the Orphanin FQ receptor the potential to bind opioid ligands through changing critical amino acids. By altering just four amino acid residues, the receptor was transformed into a kappa-like receptor capable of binding $\kappa$-selective opioids. However, intolerance for the other endogenous opioids still persisted (Meng et al., 1996). The phylogenetic analyses provide insight as to why this situation might exist. The ORL receptor did not show any direct relationship to the delta or the mu receptor in any of the phylogenetic trees including the last tree which helped to solidify the relationship among these receptors. It appears that the ORL receptor is very distantly related to the delta and mu receptors. Because there is such a distinct relationship between ORL with KOR and DOR with MOR, the mystery behind how these receptors evolved becomes increasingly more significant.
(a). Vertebrate KOR ECL 2

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(b). Vertebrate MOR ECL 2 & 3

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<td>Sturgeon</td>
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**Figure 24 ECL’s in Vertebrate Alignments:** Vertebrate amino acid alignments of extracellular loops for each receptor. Sequences were aligned using ClustalW and shaded regions show conserved amino acids.
3. Genome Duplication

The high conservation that exists between MOR and DOR versus KOR and ORL-1 may be a result of two whole genome duplications (WGD). In 1970 Susumu Ohno came forth with his 2R hypothesis which proposed that the vertebrate genome was exposed to two rounds of WGD. A genome duplication was thought to take place after the introduction of urochordates such as the tunicate, but before the radiation of jawed vertebrates. When a genome duplication occurs, one copy of the gene retains functionality while the second copy is free from selective pressure to maintain the same function and can therefore evolve new functions. The HOX gene family provides evidence for the 2R hypothesis in cephalochordates and mammals. In amphioxus there is a single HOX cluster while humans have a cluster of four HOX genes all of which are located on different chromosomes (reviewed in Kasahara 2007). If this gene family was duplicated by a single gene duplication event, the ancestral gene and all of its derived genes would reside on the same chromosome. However, when the entire genome is duplicated, the new set of chromosomes is subject to mutations, translocations, chromosomal rearrangement and deletions. Both the ligands and receptors of the opioid/orphanin gene family exhibit patterns of WGD.

The four opioid/orphanin ligands, enkephalin, dynorphin, orphanin, and \( \beta \)-endorphin, are encoded by four different precursors, proenkephalin, prodynorphin, proorphanin, and proopiomelanocortin (POMC) respectively. It is believed that these four precursors evolved from a single ancestral proenkephalin-like gene through successive duplication events. Each duplication event is thought to give rise to two copies of the proenkephalin-like gene. One copy would retain its original proenkephalin-like function
while the other copy either became a pseudo gene or a novel gene. The ancestral proenkephalin-like gene first gave to POMC, the proorphanin, and finally prodynorphin. The duplication events are outlined in Figure 25 (reviewed in Dores et al., 2002). The protochordate duplication gave rise to proorphanin and the agnathan duplication gave rise to the most recent precursor, prodynorphin. Since prodynorphin arose after the agnathan duplication, it should not be present within the jawless vertebrates. However, this study reveals that prodynorphin is in fact present within the lamprey. As the lamprey genome was probed with a proenkephalin sequence, a prodynorphin sequence was pulled out. That sequence was used to design primers and a portion of the lamprey prodynorphin sequence was cloned. This portion included a part of dynorphin A and dynorphin B shown in Figure 26. Therefore, the evolutionary scheme presented in Figure 25 needs to be adjusted. It now appears more likely that the prodynorphin gene is ancestral to the proenkephalin gene. This begs the questions, what is the ancestral opioid gene, and do the opioid/orphanin receptors follow the same duplication pattern as their ligands?
Figure 25: Proposed evolution of the opioid/orphanin gene family. Abbreviations:

POC = proopiocortin, POMC = proopiomelanocortin (Dores et al., 2002)
Figure 26 Lamprey Prodynorphin: Amino acid and nucleotide prodynorphin sequence pulled from the lamprey genome. The area where the primers were designed is underlined and the cloned fragment is highlighted. The brackets enclose α-neoendorphin, dynorphin A, and dynorphin B respectively. The sequence can be found using the Contig 30377 in the Ensembl genome database.
A study by Dreborg et al (2008) attempted to answer this question. They carried out phylogenetic analyses on the opioid receptor gene family and twenty neighboring gene families. Using relative dating, they found that the opioid receptors along with the neighboring gene families seem to have expanded in two tetraploidizations early in vertebrate evolution. The genomic regions that were analyzed in the Dreborg study share common evolutionary history in that they consist of quartets of chromosomes from each duplication event. This is why the opioid receptor gene family, along with the some of the other neighboring gene families consist of four members. The vertebrate opioid system was already relatively complex before the radiation of jawed vertebrates. Based on sequence identity, chromosomal location, and pharmacological studies, the mu and delta receptors are more closely related to each other and the kappa and ORL receptors are more closely related to each other (Dreborg et al., 2008). The receptors cloned in this study also support these findings both on a phylogenetic basis and on a sequence identity basis. Based on this, a proposed model for the evolution and divergence of the opioid receptor gene family is presented in Figure 27.
Figure 27: Proposed evolution of the opioid/ORL1 receptor gene family (Lecaude 2004)
4. Future Studies

The initial steps in this study to clone opioid/orphanin receptors out of extant members of some of the oldest vertebrates is certainly a step in the right direction, but to bring the characterization of these receptor sequences to completion, the 5' and 3' ends of each receptor need to be found. Each species should also be screened for any possibility of other opioid/orphanin receptors. It is interesting to note that the degenerate primer set used to pull out the LR DG, HS DOR, WS KOR, and WS ORL fragments were capable of pulling out any of the four opioid/orphanin receptors. However, the degenerate primer set repeatedly pulled out only delta-like and mu-like fragments with lamprey, delta-like fragments with horn shark, and kappa-like and ORL-like fragments with white sturgeon. This raises curiosity and highlights the significance about the existence of other opioid/orphanin receptors within these animals.

The lamprey prodynorphin cloning needs to be completed as well. Obtaining full length prodynorphin sequence within the lamprey will certainly change the evolutionary beliefs about the opioid/orphanin gene family in the science world. Cloning this crucial piece of the puzzle will provide evidence that may clear up questions raised around the order in which the opioid/orphanin ligands evolved.

Cloning opioid/orphanin receptors out of lamprey, horn shark, and white sturgeon certainly provides insight about receptor structure but to fully understand how these new receptors contribute to this receptor family, binding studies should be undertaken to reveal the pharmacological profile of each new receptor. If all four of these receptors can be cloned and characterized from each species presented in this study, it would offer a great deal of information not only about the evolution of the receptors alone, but also the
co-evolution of the opioid/orphanin gene family as a whole. Provided with evolutionary knowledge about the opioid/orphanin receptors in non-mammalian vertebrates will help to better understand analgesic mechanisms in humans by identifying crucial amino acids that determine type selectivity of opioid analgesics. Using non-mammalian vertebrate models for analgesic research can be used to enhance the understanding of opioid drug selectivity (Stevens, 2008). Therefore, completing the cloning of the opioid/orphanin receptors in each of the species within this study is crucial to pain and analgesic research.
References


