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Title: An Opioid-Like Receptor in the Roughskin Newt, *Taricha granulosa.*

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Frank L. Moore

The main objectives of the current study were to obtain the complete cDNA sequence of an opioid-like receptor from an amphibian, the roughskin newt, *Taricha granulosa*, and investigate the receptor's tissue distribution and regulation following chronic exposure to the glucocorticoid corticosterone (CORT).

Degenerate primers designed in highly conserved regions of characterized opioid receptors were used to amplify opioid-like receptor fragments from a newt brain cDNA library. Receptor fragments with high sequence identity to the orphanin opioid receptor type, also termed the 'opioid receptor-like' (ORL1) receptor, were selected for 3' and 5' RACE (rapid amplification of cDNA ends) reactions to obtain the full-length receptor cDNA sequence. By this approach, we obtained a cDNA sequence that putatively encodes a 368 amino acid protein with high sequence identity (57%) to the human ORL1 receptor. Therefore, hereafter we refer to this receptor as the newt ORL1-like (nORL) receptor. The nORL receptor also has identity with the mammalian kappa (κ) opioid receptor at a number of residues that may enable it to recognize both ORL1- and κ- receptor selective ligands.

The tissue distribution of the nORL receptor was determined by reverse-transcriptase polymerase chain reaction (PCR). RNA from a variety of tissues was reverse-transcribed into cDNA using an oligo-dT primer, and the resultant cDNA

was used as template in PCR reactions with nORL receptor-specific primers. Appropriately sized amplicons were produced in reactions with cDNA template originating from newt brain, spinal cord, and lungs. No amplification occurred in reactions with template cDNA from newt spleen, small intestine, heart, liver, sperm duct, bladder, or kidney.

The regulation of the nORL receptor following chronic exposure to the glucocorticoid corticosterone was investigated using real-time PCR. Animals were exposed continuously to CORT for 10 days using surgically implanted Silastic capsules packed with CORT powder. Control animals received empty Silastic capsules, or no treatment. The relative quantitation of the nORL receptor messenger RNA (mRNA) was achieved by real-time PCR, and mRNA levels for the hormone-treated animals were compared to those of the controls. The same samples were used for the relative quantitation of intracellular glucocorticoid receptor (iGR) mRNA. There was no change in the expression of mRNA for the nORL receptor or the iGR following chronic exposure to CORT as compared to the controls.

In conclusion, this study provides evidence for an opioid-like receptor in the roughskin newt that has high sequence identity to the mammalian ORL1 opioid receptor. To the best of our knowledge, this is the first complete opioid receptor cDNA sequence obtained for an amphibian. The nORL receptor appears to principally function in central nervous system (CNS) processes in the newt, as evidenced by its primary localization to brain and spinal cord. The role of the nORL receptor in the periphery may be limited to a function in the lungs, and awaits further investigation. The current study was also the first to investigate the effects of a stress hormone on the regulation of an opioid receptor in an amphibian. Our results indicate that chronic exposure to the stress hormone corticosterone does not impact the levels of nORL receptor or intracellular glucocorticoid receptor mRNA in the newt spinal cord.

An Opioid-Like Receptor in the Roughskin Newt, *Taricha granulosa*

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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Eliza A. Walthers, Author

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CHAPTER 1:

THESIS INTRODUCTION

Previously, it was hypothesized that a membrane corticosterone binding site in the roughskin newt, *Taricha granulosa*, has the molecular identity of an opioid-like receptor (Evans et al. 2000b). Obtaining the full-length cDNA sequence of a newt opioid-like receptor is a step towards testing this hypothesis. Subsequently, experiments to characterize the relationship of the newt opioid-like receptor to corticosterone can be designed. Therefore, the goals of the present study were to obtain the complete cDNA sequence for a newt opioid-like receptor, and to conduct a preliminary investigation on the relationship of this receptor to the glucocorticoid corticosterone. The following introduction was designed to summarize the research leading up to the present thesis, provide background on the opioid system, and specifically highlight characteristics of the type of mammalian opioid receptor that shares the highest identity to the newt opioid-like receptor obtained in the present study. Therefore, this introduction includes: 1) a description of the research concerning the characterization of the newt membrane corticosterone receptor (mCR); 2) the development of the hypothesis that the mCR may be an opioid-like receptor; 3) a historical overview of the endogenous opioid system; 4) a section highlighting the characteristics of the mammalian opioid receptor-like (ORL1) receptor type; 5) a section describing the proposed evolution of the opioid system, and the phylogenetic distribution of the endogenous opioid peptides and receptor types; and 6) a section dedicated to opioid receptors and peptides in amphibians.

EVIDENCE FOR A NEWT NEURONAL MEMBRANE CORTICOSTERONE RECEPTOR

In the classic genomic model for steroid hormone action, steroids affect cell physiology by modulating gene transcription. Steroid hormones diffuse across the cell membrane due to their lipophilic nature, and bind to intracellular receptors that function as ligand-dependent transcription factors. The steroid-receptor complex binds to response elements in the promoter regions of select genes, and regulates their transcription (Norris, 1997). Effects from changes in gene transcription generally take at least 20 minutes to manifest, so the genomic model does not account for the rapid effects (milliseconds to minutes) of steroids (for review see Moore & Evans, 1999), suggesting an additional signaling pathway for steroid hormones.

The rapid inhibition (< 8 min) of male newt (*Taricha granulosa*) courtship behavior in response to systemic injections of corticosterone (CORT) or applied stress is an example of a steroidal effect that occurs too rapidly to be explained by a transcriptional mechanism (Moore & Miller, 1984; Orchinik et al. 1991). In agreement with this behavioral observation, neurophysiological studies demonstrate that CORT rapidly inhibits the activity of medullary neurons (Rose et al. 1993, 1995). Rapid effects on cell physiology can be brought about by membrane receptor-mediated signaling systems coupled to cellular second messengers. Therefore, the observed rapid effects of CORT could be due to signal transduction through a membrane receptor.

Newt neuronal membranes have a high affinity (K_d = 0.5 nM), specific, and saturable [^{3}H]CORT binding site that is protein in nature (Orchinik et al. 1991). These characteristics meet some of the criteria for membrane-associated receptors. The only other steroid with high affinity for this site is cortisol (K_d = 3.75 nM); other steroids exhibit little or no ability to inhibit [^{3}H]CORT binding to the membrane site (Orchinik et al. 1991). Furthermore, there is a high correlation between the ability of CORT and tested steroids to inhibit clasping (behavioral

response), and their ability to inhibit [^{3}H]CORT binding to the membrane site (Orchinik et al. 1991). These results indicate a membrane corticosterone receptor (mCR) is present in the newt that mediates the rapid effects of CORT on behavior.

Further characterization of the mCR demonstrated that guanyl nucleotides inhibit, while Mg^{2+} enhances, the binding of [^{3}H]CORT to the membrane site (Orchinik et al. 1992), characteristics of a G-protein coupled receptor (GPCR). The eventual solubilization, partial purification and biochemical characterization of the mCR revealed that it is an acidic glycoprotein with a molecular weight of ~63 kDa (Evans et al. 1998, 2000a). However, the molecular identity of the mCR remains unknown.

MOLECULAR IDENTITY OF THE mCR: POSSIBILITY THAT IT IS AN OPIOID-LIKE RECEPTOR

A previous study demonstrated that the steroid progesterone is capable of binding to and inhibiting the activity of a known GPCR, the oxytocin receptor (Grazzini et al. 1998). To investigate the possibility that the mCR is also a known GPCR, an experiment was conducted to evaluate the ability of the mCR to recognize non-steroid ligands that mediate behavioral responses similar to those caused by CORT, and whose cognate receptors are members of the GPCR superfamily (Evans et al. 2000b). Of the ligands tested, only five are capable of displacing [^{3}H]CORT in a competition assay, and all are opioids: the endogenous kappa-selective agonist dynorphin 1-13 amide, the synthetic kappa-specific agonist U50,488, the non-selective opioid antagonist naloxone, and the kappa agonists bremazocine (BRE) and ethylketocyclazocine (EKC). However, four opioids that display affinity for kappa receptors in mammals are not competitive at the newt [^{3}H]CORT binding site: the kappa-specific synthetic agonist U69,593, the kappa-specific antagonist nor-BNI, the non-selective opioid antagonist diprenorphine, and the non-selective agonist etorphine. Based on these results, it appears that the

molecular identity of the [^{3}H]CORT binding site might be related to a kappa-like opioid receptor.

In support of this hypothesis, CORT and kappa opioids have overlapping behavioral and physiological effects. Behavioral similarities in the newt include the inhibition of male courtship behavior, and the suppression of locomotor activity, by both CORT (Moore & Miller, 1984; Chiavarini, 1997) and kappa-selective agonists (Deviche & Moore, 1987; Deviche et al. 1989; Lowry et al. 1990). As endocrine regulators, both CORT and kappa agonists decrease adrenocorticotropic hormone (ACTH) activity by negatively modulating secretions of corticotropin-releasing hormone (CRH) and vasopressin from the paraventricular nucleus (PVN) of the hypothalamus (Wells et al., 1991; Currie et al. 1994; Liu et al. 1995; Edwardson & Bennett 1974).

Although not conclusive, these observations support the hypothesis that CORT is acting through an opioid-like GPCR to rapidly affect behavior. In order to test this hypothesis, the molecular identities of the opioid-like receptors in the roughskin newt have to be revealed.

THE OPIOID SYSTEM: PEPTIDES AND RECEPTORS— AN HISTORICAL PERSPECTIVE

Alkaloids are chemicals that are produced by plants, and when taken in high concentrations can affect the mood and behavior of humans. Alkaloids were first used for medicinal purposes some 4,000 years ago, when poppies (*Papaver somniferum*) were cultivated for the liquid found in their unripe seed capsules. This liquid, known today as opium, may have been used in religious rituals for its ability to evoke euphoria. Eventually, morphine was isolated as the active substance of opium, and was subsequently introduced into medicine for the treatment of severe or chronic pain.

The isolation of exogenous opiates such as morphine generated interest in the probable existence of an endogenous opioid system, consisting of receptors and specific ligands. The first evidence for specific opiate binding sites came when three groups simultaneously demonstrated the existence of high-affinity, saturable, stereospecific binding sites in brain membranes for the exogenous opiates [^{3}H]naloxone or [^{3}H]etorphine (Pert & Snyder, 1973; Simon et al. 1973; Terenius 1973). A few years later, the first evidence for the existence of several types of opioid receptors was demonstrated by the pharmacological actions of various opiates and their derivatives in dogs (Gilbert & Martin, 1976; Martin et al. 1976), and by the discovery of a high affinity receptor for enkephalins in the mouse vas deferens (Lord et al. 1977). The opioid-binding sites were pharmacologically classified into three main types, referred to as mu (μ), delta (δ), and kappa (κ) receptors.

At the same time, research was focused on the isolation of endogenous opioid ligands. In 1975, two pentapeptides were isolated from pig brain—Leu-enkephalin (YGGFL) and Met-enkephalin (YGGFM) (Hughes et al. 1975). This was followed by the isolation of β-endorphin from camel pituitary glands (Li & Chung, 1976), and dynorphin from porcine pituitary (Goldstein et al. 1981) and duodenum (Tachibana et al. 1982). Following the isolation of these endogenous opioid peptides, the cloning of the cDNA for their precursor proteins was undertaken. Three precursor proteins for the opioid peptides were discovered; Leu- and Met-enkephalin derive from preproenkephalin (Noda et al. 1982), β-endorphin is a product of proopiomelanocortin (POMC)—which also gives rise to ACTH and α-melanocyte stimulating hormone (Nakanishi et al. 1979), and dynorphin is a cleavage product of preprodynorphin (Kakidani et al. 1982). In terms of receptor selectivity, β-endorphin binds to μ and δ receptors with comparable affinities, while Met- and Leu-enkephalins are considered endogenous ligands for δ receptors, and dynorphin for κ receptors.

The first cloning of an opioid receptor did not occur until almost 20 years after the initial radioligand binding studies had been performed on brain membranes. In 1992, two groups employed the technique of expression cloning to isolate the cDNA for the mouse δ receptor (mDOR) (Evans et al 1992; Kieffer et al 1992). Following the isolation of the mDOR, low stringency screening using a probe based on the mDOR resulted in the isolation of the cDNA for the mouse κ receptor (Yasuda et al, 1993) and the rat μ receptor (rMOR) (Chen et al. 1993). Since the first receptors were cloned, many groups have cloned the receptors from a variety of species and tissues (reviewed in Kieffer, 1995).

The continued use of low stringency hybridization procedures eventually lead to the isolation of a clone that had high sequence identity to the three opioid receptors, but which did not bind opiates or endogenous opioids with high affinity (Mollereau et al. 1994; Fukuda et al. 1994; Bunzow et al. 1994; Chen et al. 1994; Wang et al. 1994; Wick et al. 1994; Nishi et al. 1994; Lachowicz et al. 1995; Halford et al. 1995). The protein, now referred to as the opioid receptor-like (ORL1) receptor, took its place among the orphan GPCRs. The endogenous ligand for the ORL1 receptor was isolated by two groups using reverse pharmacology almost two years later, and was called nociceptin by one group (Meunier et al. 1995), and orphanin FQ (oFQ) by the other (Reinscheid et al. 1995). The organization and sequence of the precursor gene for oFQ, proorphanin (Nothacker et al. 1996; Mollereau et al. 1996; Houtani et al. 1996; Saito et al. 1995), is similar to the precursor genes for the opioid peptides. Based on the sequence identity between the ORL1 receptor and the opioid receptors, and organizational similarities between proorphanin and the opioid peptide precursors, the ORL1 receptor and the oFQ peptide were tentatively classified as part of the opioid family.

THE ORL1/ORPHANIN FQ SYSTEM: MOLECULAR CHARACTERISTICS AND BEHAVIORAL IMPLICATIONS

The ORL1 receptor has seven hydrophobic regions that have high sequence identity to the membrane spanning domains of G-protein coupled receptors, and hence, like the classic opioid receptors, is classified as a GPCR. The ORL1 receptor shares the greatest sequence identity with the μ-, δ-, and κ- opioid receptors in these regions, with >80% identity in the second, third, and seventh putative transmembrane domains (TMD) (Mollereau et al. 1994). The high degree of similarity in the TMDs supports the notion that the ORL1 receptor contains a transmembrane binding pocket that is the structural equivalent of the alkaloid binding pocket of the classical opioid receptors.

The degree of sequence identity between the ORL1 receptor and the classic opioid receptors is also high in the cytoplasmic loops. The cytoplasmic loops are proposed to be the receptor sites responsible for G protein coupling, and it appears the ORL1 receptor and the opioid receptors do couple to the same class of G proteins (G_i/G_o) (for review see Hawes et al. 2000; Law et al. 2000). This class of G protein is associated with second messengers that generally act to reduce cellular excitability and inhibit neurotransmitter release, such as decreases in cAMP, enhancement of an outward K^+ conductance, and the closure of voltage sensitive Ca^{2+} channels. These cellular effects confer the ORL1 receptor, like the opioid receptors, with a largely inhibitory role in the central nervous system (CNS) circuitry.

The sequence identity between the ORL1 receptor and the opioid receptors is lowest in the N-terminus, and the second and third extracellular loops (Mollereau et al. 1994). The extracellular loops are believed to be involved in receptor selectivity, and may enable a receptor to differentiate between closely related ligands. Studies with receptor chimeras have suggested that the second extracellular loop (EL2) of the ORL1 receptor is an absolute requirement for activation by its endogenous ligand, oFQ (Mollereau et al. 1999).

The EL2 of the ORL1 receptor is highly acidic in nature, a characteristic that is only shared by the EL2 of the κ opioid receptor (KOR). However, unlike the ORL1 receptor, the EL2 of the KOR does not appear to be critical for its activation (Mollereau et al. 1999). A bifunctional chimera of these two receptors, consisting of the ORL1 sequence from the N-terminus to the start of TMD3, plus the ORL1 EL2, with the remainder consisting of KOR sequence, is capable of recognizing oFQ and dynorphin equally (Mollereau et al. 1999). These studies highlight the structural relatedness of the ORL1 and κ opioid receptors, while demonstrating that distinct receptor regions are required for receptor activation by their respective ligands (Lapalu et al. 1998).

Orphanin FQ, the endogenous ligand for the ORL1 receptor, is a heptadecapeptide with a highly positively charged core, and resembles the endogenous ligand for the KOR, dynorphin A. The precursor protein for oFQ, proorphanin, shares organizational and sequence similarities with the opioid peptide precursors, suggesting a common evolutionary origin. However, unlike all classical endogenous opioid peptides, oFQ has a phenylalanine at its N-terminus rather than a tyrosine, changing the canonical opioid tetrapeptide sequence from Y^1GGF to F^1GGF. This is a significant difference, since the N-terminal tyrosine of the classical opioid peptides is considered essential for their activity, and is reflected by the inability of oFQ to bind to or elicit a response from the μ-, δ-, or κ-opioid receptors (Meng et al. 1996; Reinscheid et al. 1995). The first amino acid of the oFQ peptide is also crucial for its activity at the ORL1 receptor, as the removal of Phe^1 from the oFQ peptide dramatically decreases (>2000-fold) its affinity and biological activity (Butour et al. 1997). However, Tyr^1 can be substituted for Phe^1 in the oFQ peptide, creating an oFQ analog that is nearly as potent as the original peptide at the ORL1 receptor (Butour et al. 1997; Reinscheid et al. 1996); thus, an aromatic ring at position one appears to be important for recognition of the oFQ peptide by the ORL1 receptor. The synthetic Tyr^1-oFQ analog does not show activity towards the classic opioid receptors unless truncated at its carboxyl

terminus, indicating the carboxyl half of the mammalian peptide may contain a domain that contributes to its exclusion from the opioid receptors (Reinscheid et al. 1998).

The shortest oFQ peptide fragment with the same affinity, biological potency, and selectivity as oFQ1-17 at the ORL1 receptor is oFQ1-13-amide. It is suggested that the positively charged core of oFQ (amino acids 8-13) is required for its biologic activity (see Meunier et al. 2000). This is in contrast to dynorphin A, the most closely related opioid peptide to oFQ, whose capacity to induce a biological response is contained within its N-terminal YGGF sequence. In this respect, oFQ apparently does not conform to the structure-activity relationship proposed for the classic opioid peptides in the 'message-address' hypothesis for receptor selectivity and activation (Chavkin & Goldstein, 1981).

In the 'message-address' hypothesis for opioid peptide selectivity and potency, the C-terminal of the peptide primarily serves a locator function ('address'), while the N-terminal YGGF sequence contains the capacity to induce a response (the opioid 'message'). However, based on the above observations regarding the structural architecture of the oFQ peptide, it appears the 'message' and 'address' domains of the peptide are reversed. Therefore, the FGGF may serve a locator function, binding to the vestigial opioid binding pocket of the ORL1 receptor, while the peptide's selectivity and activator functions are located within the core of the peptide (Meunier et al. 2000; Butour et al. 1997).

A 3-D model of an oFQ/ORL1 complex has been described and is consistent with the structural observations of both the receptor and its ligand described above (Topham et al. 1998). In the model, the two aromatic rings of the N-terminal tetrapeptide (FGGF) of oFQ are proposed to bind in the bottom of a cavity formed by transmembrane helices 3, 5, 6, and 7 of the ORL1 receptor. Residues 5-7 of the oFQ peptide, thought to contribute significantly to ORL1 receptor selectivity (Lapalu et al. 1997), end up at the EL2-TMDIV interface, which is a largely unconserved region of the receptor. Finally, the cationic core (residues 8-13) of

oFQ is in a position to establish multiple contacts with the acidic EL2 of the ORL1 receptor.

The differences in the functional architecture of the ORL1 receptor and its peptide, versus the classical opioid receptors and peptides, shed some light on its unique pharmacological profile. Initial studies on the pharmacology of the ORL1 receptor, prior to the isolation of the oFQ peptide, demonstrated its ability to bind and/or respond to some non-selective opioid agonists and antagonists. Etorphine, a potent non-selective opioid agonist, was the first opioid ligand found to be capable of activating the ORL1 receptor; however, activation required doses three orders of magnitude higher than those necessary to produce effects at the classic opioid receptors (Mollereau et al. 1994). Similarly, the antagonist diprenorphine was capable of reversing the etorphine-induced effect, but also at doses much higher than those required for μ–, δ–, or κ receptors (Mollereau et al. 1994). Another nonpeptide ligand shown to have some affinity for the ORL1 site is naloxone benzoylhydrazone, which acts as a mixed agonist-antagonist at the opioid receptors (Pan et al. 1996). Perhaps the most potent nonpeptide ligand discovered to date that acts as an agonist at the ORL1 receptor is lofentanil, which is less potent than oFQ by only a factor of ten (Butour et al. 1997). Recently, buprenorphine, an opioid with both μ- and κ- receptor selectivity, was identified as a full agonist of the ORL1 receptor by a reporter gene assay in CHO cells (Wendt et al. 1999). However, most of the traditional non-selective opioid ligands, including naloxone, are not capable of binding/activating the ORL1 receptor (Butour et al. 1997). Of the opioid peptides, only dynorphin A has substantial apparent affinity ($K_i < 1\mu M$) for the ORL1 receptor, although this affinity is still 2-3 orders of magnitude lower than that displayed at the classic opioid receptors (Butour et al. 1997). In conclusion, although the ORL1 receptor does appear to retain some ability to recognize opioid ligands, the pharmacology of the ORL1 receptor displays little similarity to that of the classical opioid receptors.

The ORL1 receptor is widely distributed throughout the CNS, including numerous regions of the forebrain, brainstem, and the dorsal and ventral horns of the spinal cord (see Mollereau & Mouledous, 2000). The mammalian ORL1 receptor is also found in select tissues of the periphery, such as the intestine, liver, vas deferens, spleen, and heart in rat (Wang et al. 1994; Dumont & Lemaire, 1998;), and kidney in pig (Osinski et al. 1999). Also, the ability of the oFQ peptide to inhibit contractions of rat bladder (Guiliani et al. 1998) and guinea pig bronchus (Rizzi et al. 1999), infers the presence of the ORL1 receptor in these organs. The distribution of the ORL1 receptor throughout the CNS and the periphery mirrors its involvement in a broad array of physiological effects.

Since their discoveries, the role of the oFQ/ORL1 and classic opioid systems in nociception, or pain perception, has been the subject of intense investigation. Based on the structural similarities between these two systems, it was originally thought that oFQ, like the classic opioid peptides, would serve a primarily analgesic function in response to painful stimuli. Therefore, researchers were surprised when initial behavioral assays suggested that just the opposite was true: oFQ administered intracerebroventricularly (i.c.v) to mice evoked a hyperalgesia—or a reduced tolerance to pain (Meunier et al. 1995; Reinscheid et al. 1995). This effect is no longer apparent in ORL1 receptor-deficient homozygous mice, indicating that it is mediated through the ORL1 receptor (Nishi et al. 1997). The apparent hyperalgesic effects of the oFQ peptide were later explained as a reversal of the stress-induced analgesia associated with the trauma of i.c.v. injection, and mediated by the endogenous opioid system (Mogil et al. 1996a). For instance, animals injected with the oFQ peptide have the same response in three behavioral assays (hot plate, tail withdrawal, and mouse writhing) as uninjected animals, whereas animals receiving i.c.v. saline injections have a reduced nociceptive response (increased pain tolerance), presumably mediated by the endogenous opioid system because the response can be blocked by naloxone (Mogil et al. 1996a). In addition, oFQ is capable of reversing analgesia induced by systemically

administered morphine (Mogil et al. 1996a), as well as opioid receptor type-selective (μ, δ, and κ) agonists given i.c.v. (Mogil et al. 1996b). Thus, the oFQ peptide potentially acts as an anti-opioid supraspinally. However, at the level of the spinal cord, oFQ does appear to be endowed with some analgesic properties. In the rat, intrathecally administered oFQ has been shown to induce analgesia (Hao et al. 1997), and to potentiate the analgesia induced by subcutaneously administered morphine (Tian et al. 1997).

Because the classical opioid receptors and the ORL1 receptor couple to the same cellular effectors, the observed differences between the opioid peptides and oFQ on nociceptive processing may be due to differences in the localization of their respective receptors throughout the neuronal circuitry (for reviews see Darland et al. 1998; Mogil & Pasternak, 2001).

In recent years, the involvement of the oFQ/ORL1 system in numerous physiological processes, in addition to its role in nociception, has become apparent. The oFQ peptide increases food intake in satiated rats (Pomonis et al. 1996), increases or decreases locomotion depending on dose (Reinscheid et al. 1995; Florin et al. 1996), and impairs spatial learning (Sandin et al. 1997). In addition, oFQ appears to play a role in the modulation of the stress response. Mice lacking the oFQ gene have higher basal plasma corticosterone concentrations than control mice, and a reduced ability to adapt to repeated stressors (Koster et al. 1999). In a seemingly related behavioral modulatory role, oFQ administered i.c.v. to rats and mice has anxiolytic-like effects on behaviors associated with stressful conditions (Jenck et al. 1997). These highlighted studies represent a small sampling of the literature to date regarding the physiological effects of oFQ (for a complete review see Mogil & Pasternak, 2001).

In conclusion, the oFQ/ORL1 system appears to be related to the classical opioid system. However, the structural and functional divergence of the oFQ/ORL1 system from the opioid system has resulted in a unique neuromodulatory system with numerous physiological roles. The elucidation of the

mechanisms of action, and the specific role of this system in the modulation of the neural circuitry, await further investigation.

OPIOID PEPTIDES AND RECEPTORS: SPECIES DISTRIBUTION

It is hypothesized that the four opioid peptide precursor genes (proenkephalin, POMC, prodynorphin, and proorphanin) arose by sequential duplication of a common ancestral gene over the past five hundred million years, and the appearance of the cognate receptor proteins (δ, μ, κ, ORL1) paralleled the divergence of the peptide genes (Douglass et al. 1984). This hypothesis has been modified to include the speculation that the ancestral opioid gene was proenkephalin-like, the first duplication produced a POMC-like gene, the second duplication resulted in a proorphanin-like gene, and the most recent duplication resulted in a prodynorphin-like gene (Figure 1.1) (Danielson and Dores, 1999).

If this hypothesis is true, the ancient animal lineages are expected to have fewer opioid peptide genes (and receptor types) than the most recent lineages. Specifically, based on the predicted duplication event time-course, the sarcopterygians (e.g. lungfish, coelacanth, tetrapods) will express all four opioid precursors; the rayfinned and cartilagenous fish will express proenkephalin, POMC, and proorphanin; the agnathans (lamprey) will express proenkephalin and POMC; and the protochordates with have only proenkephalin (Danielson and Dores, 1999).

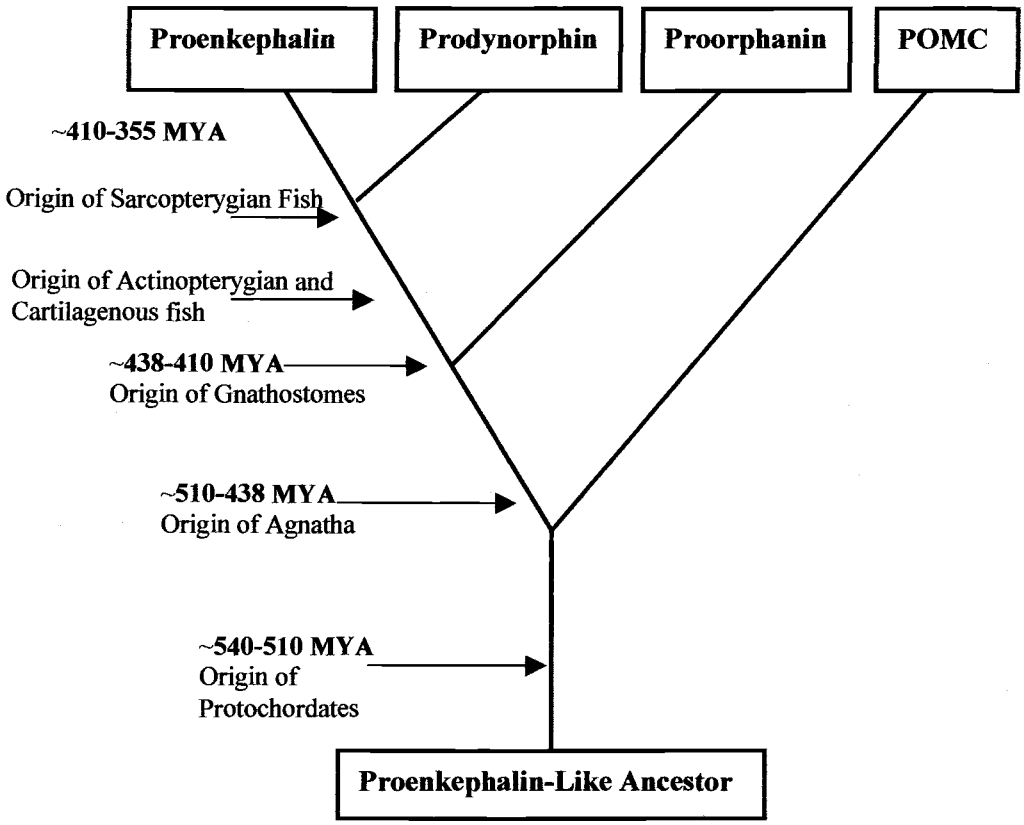
This hypothesis is supported by results from biochemical and molecular studies on the distribution of the opioid peptides across phylogeny. To date, enkephalin-related products have been detected in the broadest array of phyla, from the protochordates through the mammals (Dores et al. 1993). POMC-related products have been detected in all vertebrates tested, but their presence is questionable in invertebrates (see Danielson & Dores, 1999). And finally, it appears that

prodynorphin-related products are restricted to tetrapods (Dores et al. 1993). To date, only mammalian tissues have been biochemically screened for proorphanin-related products.

In addition to biochemical studies, molecular studies can be used to test the above hypothesis, and obtaining cDNA sequences ensures a more specific gene detection method than biochemical studies can provide. However, complete cDNA sequence information on opioid peptide genes from multiple taxa is far from complete. Thus far, only POMC cDNA has been cloned from a wide diversity of animal taxa, including agnathans, actinopterygians, amphibians, and mammals (see Danielson & Dores, 1999). The complete cDNA sequences for proenkephalin, prodynorphin, and proorphanin are almost entirely from mammals. In the case of proenkephalin, complete cDNA sequences have also been obtained for lungfish, and partial sequences are available for amphibians, zebrafish, and sturgeon (Genbank). The only complete non-mammalian proorphanin cDNA sequence has come from sturgeon (Danielson et al. 2001), and no complete non-mammalian prodynorphin sequence has been reported, although a partial sequence is available for *Bufo marinus* (Genbank).

The situation regarding complete cDNA sequences for the opioid receptors is similar. The full-length cDNA sequences for all four mammalian opioid receptor types have been obtained (Genbank). Besides mammals, full-length cDNA receptor sequences have only been submitted for zebrafish (δ, μ, ORL1) and white sucker (μ) (Genbank). However, the amplification of genomic DNA using degenerate opioid receptor primers suggests the presence of multiple opioid receptor types in chick (δ, μ, ORL1), frog (δ, μ, κ), bass (δ, μ, κ), and shark (δ, μ, ORL1) (Li et al. 1996). In addition, partial fragments for the multiple receptor types have been detected in ancient lineages such as hagfish (δ- and μ- like) (Li et al. 1996) and white sucker (δ-, κ-, and ORL1-like) (Darlison et al. 1997). These data indicate multiple receptor types, like the opioid peptides, arose early in vertebrate evolution.

Figure 1.1: Sequential duplication of a proenkephalin-like ancestral opioid gene, at distinct points over the last five hundred million years, gave rise to the four opioid peptide genes (figure adapted from Danielson and Dores, 1999). It is hypothesized that the first duplication event resulted in POMC, the second in proorphanin, and the third and most recent in prodynorphin. MYA = million years ago.



THE OPIOID PEPTIDES AND RECEPTORS IN AMPHIBIANS

As stated above, the complete POMC cDNA sequence has been obtained from an amphibian, in addition to partial sequences for proenkephalin and prodynorphin (Genbank). If the opioid peptides and their cognate receptors evolved in parallel, then the definitive presence of these three opioid peptide genes predicts the presence of *at least* three opioid receptor types in amphibians, namely the μ-, δ-, and κ- like receptors. In support of this, partial fragments for these three receptor

types have been obtained from bullfrog (*Rana catesbeiana*) genomic DNA (Li et al. 1996).

Prior to the advent of this receptor sequence information, amphibians were predicted to have multiple opioid receptor types based on pharmacological studies. Early binding assays on toad and frog brains indicated the presence of μ–, δ–, and κ–like opioid binding sites, and estimated the predominant form of opioid receptor to be κ–like (60-70%), with considerably less μ– and δ–like sites (20-30%) (Simon et al. 1982, 1984). In addition to these early studies, numerous reports have characterized multiple opioid-like receptor types (μ, δ, κ) and subtypes (κ1, κ2) in amphibians (Ruegg et al. 1980, 1981; Simon et al. 1985, 1987; Benyhe et al. 1990, 1992, 1994; Borsodi et al. 1986; Mollereau et al. 1988; Makimura et al. 1988; Wollemann et al. 1994). The pharmacological profiles of these amphibian opioid binding sites differ from their mammalian counterparts, with the κ-like site displaying a greater affinity for μ- and δ- selective opioids, and a lesser affinity for κ-selective opioids, compared to the mammalian site (Mollereau et al. 1988; Simon et al. 1982).

In addition to the pharmacological evidence, behavioral studies support the presence of multiple opioid receptor types in amphibians. In amphibians, receptor type-selective opioids have been implicated in the control of nociceptive, locomotor, and sex behaviors. The opiate morphine sulfate (a μ receptor agonist) is an analgesic when injected into the spinal cord of a frog, *Rana pipiens*, and the analgesia is attenuated by naloxone (Stevens & Pezalla, 1983; Pezalla, 1983). Also in frogs, spinal administration of dynorphin, beta-endorphin, or Met-enkephalin produces a potent, dose-dependent increase in nociceptive threshold (Stevens et al. 1987). In the roughskin newt, *Taricha granulosa*, the κ-agonist bremazocine reduces spontaneous locomotor activity, but has no affect on corticotropin releasing factor (CRF)-induced locomotion, whereas morphine does not affect spontaneous locomotion, but does reduce CRF-induced locomotion (Deviche et al. 1989; Lowry

et al. 1990). Morphine also influences locomotion in the frog, where it induces explosive motor behavior when administered systemically (Pezalla, 1983). Both bremazocine and ethylketocyclazocine (EKC) (κ agonists) reduce sexual activity in the roughskin newt, and in both cases the effect is reversed by naloxone (Deviche & Moore, 1987). These behavioral observations, which are based on the effects of receptor type-selective opioid agonists, support the presence of multiple opioid receptor types in amphibians.

In conclusion, pharmacological, behavioral, and molecular studies indicate that there will be multiple types of opioid-like receptors in amphibians. However, the true nature of the amphibian opioid receptors will not be clarified until the complete cDNA sequence information for the pharmacologically characterized types is obtained.

SPECIFIC AIMS

This thesis has three specific aims: 1) obtain the complete cDNA sequence for an ORL1-like opioid receptor from the roughskin newt, *Taricha granulosa*; 2) determine the tissue distribution of the newt ORL1-like (nORL) receptor; and 3) investigate the regulation of the nORL receptor following chronic exposure to the glucocorticoid corticosterone.

This thesis is arranged as two research chapters followed by a general summation. Chapter 2 establishes the full-length cDNA sequence identity of an ORL1-like receptor in the roughskin newt. In Chapter 3, the sequence information gained in Chapter 2 is used in Study A to determine the tissue distribution of the nORL receptor. This distribution information is then used in Study B of Chapter 3 to design a real-time PCR experiment to quantitate the expression of the nORL receptor following chronic exposure to corticosterone.

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CHAPTER 2:

A COMPLETE CDNA SEQUENCE FROM THE ROUGHSKIN NEWT, *Taricha granulosa*, THAT ENCODES AN ORL1-LIKE OPIOID RECEPTOR

ABSTRACT

A cDNA sequence that encodes a 368 amino acid protein was cloned from a urodele amphibian, the roughskin newt, *Taricha granulosa*. Sequence analysis indicates the deduced protein belongs to the G protein coupled receptor (GPCR) superfamily and is an opioid-like receptor. When compared to opioid receptor types from a variety of species, the newt sequence shares the greatest amino acid identity with the human opioid receptor-like (ORL1) receptor (57%), and a fairly equal degree of identity across the human κ-, μ-, and δ- receptors (48-51%). The newt ORL1-like (nORL) receptor also shares a number of key residues with the κ opioid receptor, supporting the prediction that the nORL receptor will bind a subset of orphanin FQ- and dynorphin-like ligands. In this respect it appears the newt may possess an opioid receptor that is a functional intermediate between the κ- and ORL1- receptor types characterized in mammals. To the best of our knowledge, this is the first full-length opioid receptor cDNA sequence obtained from an amphibian.

INTRODUCTION

Opioid peptides are endogenous neuromodulators involved in a diverse array of physiological functions, including a principle role in nociceptive processing. Opioid peptides act at target cells through membrane receptors belonging to the superfamily of G-protein coupled receptors (GPCR). The opioid receptors recognize both endogenous opioid peptides and exogenous nonpeptide molecules known as alkaloids or opiate drugs. Prior to cloning the genes for the opioid receptors, three major types were proposed based on pharmacological studies: kappa (κ), delta (δ), and mu (μ) (Gilbert & Martin, 1976; Martin et al. 1976; Lord et al. 1977). Almost twenty years later, the cDNA sequence for the mouse δ receptor was isolated using expression cloning (Evans et al. 1992; Kieffer et al. 1992), and low stringency screening with a probe based on this sequence resulted in the isolation of the mouse κ– (Yasuda et al, 1993) and rat μ– receptor (Chen et al 1993) cDNA sequences.

The continued use of low stringency hybridization procedures eventually lead to the isolation of a clone that had high sequence identity to the three opioid receptor types, but which did not bind opiates or endogenous opioids with high affinity (Mollereau et al. 1994; Fukuda et al. 1994; Bunzow et al. 1994; Chen et al. 1994; Wang et al. 1994a; Wick et al. 1994; Nishi et al. 1994). The protein, now referred to as the opioid receptor-like (ORL1) receptor, remained an orphan GPCR until the discovery of its endogenous ligand in 1995 by two groups (Meunier et al. 1995, Reinscheid et al. 1995).

The endogenous ligand for the ORL1 receptor was named nociceptin by one group due to its apparent hyperalgesic properties (Meunier et al. 1995), and orphanin FQ (oFQ) by the other, in reference to its orphan receptor, and the first and last amino acids of the peptide (Reinscheid et al. 1995). oFQ is a heptadecapeptide bearing a close resemblance to the endogenous ligand for the κ opioid receptor, dynorphin A. The organization of the oFQ precursor gene,

proorphanin (Nothacker et al. 1996; Mollereau et al. 1996; Houtani et al. 1996; Saito et al. 1995), indicates that it is evolutionarily related to the three opioid precursor genes: proenkephalin, proopiomelanocortin (POMC), and prodynorphin.

Despite the structural similarities between the ORL1 receptor and the 'classical' opioid receptors (μ, δ, and κ), a functional separation exists between these two systems. The ORL1 receptor does not bind with high affinity the majority of endogenous or exogenous opioids recognized by the classical opioid receptors (Mollereau et al. 1994; Butour et al. 1997). Conversely, the oFQ peptide only recognizes and activates the ORL1 receptor (Meng et al. 1996a; Reinscheid et al. 1995); this is unlike the classical endogenous opioid peptides, which show varying degrees of affinity and activity at all three (κ, δ, μ) opioid receptor types.

The ORL1 receptor and the classical opioid receptors couple to the same class of G proteins (G_i/G_o). However, the two systems often have different physiological effects, especially in the case of nociceptive processing. While the classical opioid peptides are known to induce analgesia, oFQ can reverse this effect (Mogil et al. 1996a, 1996b), and is reported to induce hyperalgesia in some instances (Meunier et al. 1995; Reinscheid et al. 1995). Therefore, although they appear to share a common evolutionary origin, these two systems have functionally diverged.

The functional isolation of the oFQ/ORL1 system from the classical opioid systems appears to be the result of minor changes in the sequence of the oFQ peptide and its receptor over time. For instance, the oFQ peptide differs from the classical opioid peptides at position one of its 'opioid core'. The 'opioid core' is the tetrapeptide (YGGF) at the N-terminus of all opioid peptides that is proposed to be responsible for receptor activation. The oFQ peptide sequence has a phenylalanine, rather than a tyrosine, at position one (FGGF). When the oFQ tetrapeptide sequence is modified by substituting a tyrosine for phenylalanine at position one, as in the classical opioids, an oFQ analog is produced that is capable of binding to and activating κ opioid receptors, while retaining its activity at the ORL1 receptor (Reinscheid et al. 1996; 1998). Conversely, by changing as few as

four residues in the ORL1 receptor to match the amino acids in the classical opioid receptors, the resultant mutant receptor can recognize prodynorphin products with high affinity (Meng et al. 1996a). These results highlight the close structural relationship between the oFQ/ORL1 system and the classical opioid system, and support the hypothesis that the peptides and receptors share common ancestors.

When did these two systems diverge? This question has been addressed by examining the distribution of the opioid peptides and their receptors (including oFQ and the ORL1) across phylogeny. Complete cDNA sequences that encode the four opioid precursors (proenkephalin, POMC, prodynorphin, proorphanin) and receptor types (δ, μ, κ, ORL1) have been obtained for mammals (Genbank). However, this sequence information for non-mammalian vertebrates is far from complete. Complete opioid receptor cDNA sequences are reported for two non-mammalian vertebrates to date, zebrafish (δ, ORL1, μ) and white sucker (μ) (Genbank). Partial cDNA sequences for κ-, δ-, and ORL1-like receptors have been obtained from the white sucker (*Catostomus commersoni*) (Darlison et al. 1997), and fragments for μ- and δ-like receptors have been cloned from hagfish (*Eptatretus stoutii*) genomic DNA (Li et al. 1996), indicating multiple opioid receptor types were present early in vertebrate evolution.

Although it appears the ORL1 receptor type is present in teleosts, there is no evidence for this receptor type in an amphibian prior to the present study. Partial genomic DNA sequences for κ-, δ-, and μ-like receptors have been obtained from the bullfrog, *Rana catesbeiana*, but no ORL1 fragments were detected in that study (Li et al. 1996). Similarly, although the proenkephalin, POMC, and partial prodynorphin cDNA sequences have been obtained from amphibians, no sequence has been obtained for the proorphanin gene in amphibians (Genbank). Therefore, although we would predict four opioid receptor types in amphibians based on their presence in more ancient lineages (e.g. teleosts), the exact nature of the amphibian opioid receptors will not be clarified unless their cDNA sequences are obtained.

In the present study we obtained the complete cDNA sequence for an amphibian opioid-like receptor. Degenerate primers, designed in highly conserved regions of opioid receptors from other species, were used to PCR-amplify opioid-like receptor fragments from a roughskin newt (*Taricha granulosa*) brain cDNA library. PCR products with high sequence identity to the mammalian ORL1 receptor were then used to design specific primers for 5' and 3' RACE (rapid amplification of cDNA ends) reactions to obtain a full-length cDNA. By this approach, we obtained a cDNA that encodes a 368 amino acid protein with a high level of sequence identity to the mammalian ORL1 receptor. However, the newt ORL1-like receptor differs from its mammalian homologue at a number of key residues that will potentially enable it to recognize both oFQ- and dynorphin-specific ligands. In this respect it appears the newt may possess an opioid-like receptor that is an intermediate between the mammalian κ- and ORL1- receptor types. This sequence provides new information on amphibian opioid receptors, and insights into the evolution of the ORL1 and opioid receptor systems.

MATERIALS AND METHODS

Degenerate primer design and cDNA library amplification

Degenerate primers were designed based on highly conserved regions common to the multiple opioid receptor types identified in diverse vertebrate phyla (Li et al. 1996). Primer names and sequences are as follows (Refer to Table 2.1 and Figure 2.1 for complete primer information):

OR5'-2: 5'-ACCAAYATYTACATHTTYAA-3'

OR3'-2: 5'-GGTRAACATRTTRTAGTARTC-3'

Primers (Gibco BRL Custom Primers, Invitrogen, Carlsbad, CA) were resuspended in ddH_2O to a concentration of 50μM. The degenerate primers were used in a polymerase chain reaction (PCR) to amplify opioid-like receptor fragments using a newt brain cDNA library as template. PCR reactions were carried out in 20 μl volumes containing 2 μl 10x PCR buffer (MBI Fermentas, Hanover, MD), 1.5 mM $MgCl_2$, 1.25 mM dNTP mix, 0.4 U Taq polymerase, 0.1-1.0 μg cDNA library, and 0.5 nmols of each primer. Reactions were placed in a thermocycler for 2 min at 94°C, followed by 35 cycles of 94°C for 30s, 50°C for 30s, and 72°C for 1 min, and concluding with a 7 min extension at 72°C. PCR products were screened for size by 1.5% agarose gel electrophoresis and ethidium bromide staining. Appropriately sized products (~152 bp) were ligated into the pGEM T-easy vector (Promega Co., Madison, WI). Ligations were transformed into JM109 *Escherichia coli* competant cells (Promega), and grown overnight at 37°C on LB plates containing ampicillin, X-gal, and IPTG.

Colonies containing ligation products were selected based on ampicillin resistance and their lack of a functional β-galactosidase gene (blue-white screening). Selected colonies were used to inoculate 3 ml of Luria Broth containing 100 μg/ml ampicillin, and were grown overnight with shaking at 37°C. Plasmid purification by alkaline lysis was performed on the bacterial cultures with

a Qiagen Miniprep Kit (Valencia, CA). Purified plasmid was quantified on a Beckman DU530 spectrophotometer (Beckman Coulter, Inc.) and then sequenced by the Central Services Laboratory at Oregon State University. Sequences were analyzed with a BLAST-N 2.0 search provided by NCBI.

Specific primer design, RNA purification, and 3'RACE

Sequences from clones that had high sequence identity to the mammalian opioid receptor-like (ORL1) receptor were used to design a newt-specific ORL1 primer for a 3'RACE (Rapid Amplification of cDNA Ends) reaction. The primer (One Trick Pony, Ransom Hill Bioscience, Inc. Ramona, CA) name and nucleotide sequence follows (see also Table 2.1 & Figure 2.1):
Orph3'Race: 5'-CTGGCTGATGCGCTGGTGCTCGT-3'

Five newts (*Taricha granulosa*) were anesthetized by placement in ice water and sacrificed by rapid decapitation. Brains were collected and flash-frozen in liquid nitrogen, and RNA was isolated according to the RNA STAT-60 protocol (Tel-Test, Inc. Friendswood, TX). Purified RNA was resuspended in RNase-free H_2O, quantified in a Beckman DU530 spectrophotometer (Beckman Coulter, Inc.), and examined with 1.5% agarose gel electrophoresis for the expected ribosomal bands (4.7 kb and 1.9 kb) and the presence of genomic DNA.

Purified total RNA (1.0 μg) was used in a first-strand 3' RACE-ready cDNA synthesis reaction using the Clontech SMART RACE cDNA amplification kit and protocol (BD Biosciences Clontech, Palo Alto, CA). The first-strand cDNA generated was then used as template in a 50 μl PCR reaction containing the Orph3'Race primer (0.2 μM) and an antisense primer from the Clontech kit, as specified in the Clontech protocol. PCR reaction products were examined by 1.5% agarose gel electrophoresis, and a band of ~1.6 Kb was identified as a potential 3'RACE gene-specific product. The band was cut from the gel and purified using a

Bio101 geneclean kit and protocol from ISCBioExpress (Intermountain Scientific Corporation, Kaysville, UT).

The purified 3'RACE product was ligated into a vector and transformed into competent cells, as described above. White colonies were screened by PCR with vector specific primers (M13F and M13R, Promega Co., Madison, WI) for the presence of an appropriately sized vector insert, and then used to inoculate overnight cultures. Purified plasmids were sequenced by the Central Services Laboratory at OSU, and sequences were analyzed for identity using NCBI's BLAST-N 2.0 search. A clone, named 3'RACE Orph, was found to contain the cDNA for the newt ORL1 receptor starting at amino acid residue 93 in transmembrane domain (TMD) 2 and continuing through the end of the coding region to the poly-A tail.

Specific primer design and 5'RACE

The sequences obtained from the initial screening and the 3'RACE clones were used to design primers specifically for 5'RACE. Two primers (Orph5'Race and INTR) were designed for use as gene-specific primers in the 5'RACE reaction. A third primer (INTF) was designed for use with INTR in nested PCR reactions to verify PCR product specificity. The Orph3'Race primer, described above, was available for use with Orph5'Race for the same purpose. The nucleotide sequences for the primers were as follows (see also Table 2.1 & Figure 2.1):

Orph5'Race: 5'-AGTCGATGGCGATTGCGATTTTGC-3'

INTR: 5'-TGCGGAAACAAGCCTTGAAGTTCTCG-3'

INTF: 5'-GTGCCCGTCATGGTGATGGGGTCT-3'

Purified total RNA (2.0 μg) was used in a first strand 5' RACE-ready cDNA synthesis reaction using the SMART RACE cDNA amplification kit and protocol provided by Clontech. The 5' RACE-ready first strand cDNA was used in a 50 μl

PCR reaction with the nORL-specific primers (either Orph5'Race or INTR, 0.2 μM), and a primer provided by Clontech according to kit specifications. The cycling parameters used were as follows: 94°C for 2 min; 5 cycles of 95°C for 20s, 72°C for 4.5 min; 5 cycles of 94°C for 20s, 70°C for 30s, 72°C for 4.5 min; 39 cycles of 94°C for 20s, 68°C for 30s, 72°C for 4.5 min; and 72°C for 5min. A 5'RACE band was identified on a gel after a PCR reaction using INTR as the nORL-specific primer, and was isolated and purified from the gel as described above. The purified fragment was screened for nORL specificity in a nested PCR reaction using INTF and INTR (cycling parameters: 94°C 2 min; 35 cycles of 94°C 10s, 70°C 30s, 72°C 1 min; 72°C 7 min). nORL specificity was confirmed, and the purified fragment was cloned and sequenced as previously described. Sequence analysis by BLAST-N 2.0 indicated that the clone contained the sequence of the nORL to within ~56 amino acids of the start codon (close to the start of TMD1). Another clone obtained by the same method, but primed by Orph5'Race, contained the nORL sequence to within ~75 amino acids of the start codon.

In the course of trying to obtain the 5'end of the nORL receptor with the Clontech kit, it became apparent that the presence of truncated mRNA in the first strand cDNA synthesis reaction was resulting in the amplification of incomplete 5' fragments during the subsequent PCR reaction. To try to alleviate this problem, we switched to the Ambion 5'RACE RLM kit (Ambion, Austin, TX). This kit was designed to ensure that only full-length mRNA reverse-transcribed into cDNA is capable of being amplified in the 5'RACE reaction; all truncated mRNA, rRNA, and tRNA are rendered incapable of being reactive through the removal of their 5'phosphate group and subsequent inability to be linked to an adaptor sequence necessary for the 5'RACE PCR. Sam Bradford designed a primer for use with the Ambion kit, and performed the 5'RACE reaction that produced a clone containing the entire 5' end of the nORL. The primer name and sequence follows (see also Table 2.1 & Figure 2.1):

5'RC_266: 5'-TTGGCTTTGTGTGGTGTACGGATGT-3'

The cycling conditions used in the successful 5'RACE reaction were: 94°C for 3 min; 4 cycles of 94°C 45s, 62°C 45s, 72°C 1 min; 34 cycles of 94°C 30s, 62°C 30s, 72°C 1 min; and 72°C for 6 min. This 5'RACE product was cloned and sequenced in a manner similar to that described above.

Sequence analysis

The identity of the cloned cDNA was determined through the use of BLAST-N 2.0 provided by NCBI. Multiple sequence alignments were performed using CLUSTALW accessed through the Biology Workbench, a service provided by the San Diego Supercomputer Center (SDSC), a National Laboratory for Computational Science and Engineering at the University of California San Diego. The dendrogram was created using CLUSTALW and Phylip's Drawtree, also accessed through the Biology Workbench.

Table 2.1: The primer names, nucleotide sequences, and corresponding nORL receptor nucleotide numberings (n.t.#) for all primers used in the isolation of the complete nORL receptor cDNA sequence.

Primer Name	Primer Nucleotide Sequence (5'-3')	n.t. #
OR5'-2	ACCAAYATYTACATHTTYAA	253-263
OR3'-2	GGTRAACATRTTRTAGTARTC	382-402
ORPH3'RACE	CTGGCTGATGCGCTGGTGCTCGT	277-299
ORPH5'RACE	AGTCGATGGCGATTGCGATTTTGC	362-385
INTF	GTGCCCGTCATGGTGATGGGGTCT	541-564
INTR	TGCGGAAACAAGCCTTGAAGTTCTCG	963-988
5'RC_266	TTGGCTTTGTGTGGTGTACGGATGT	473-497

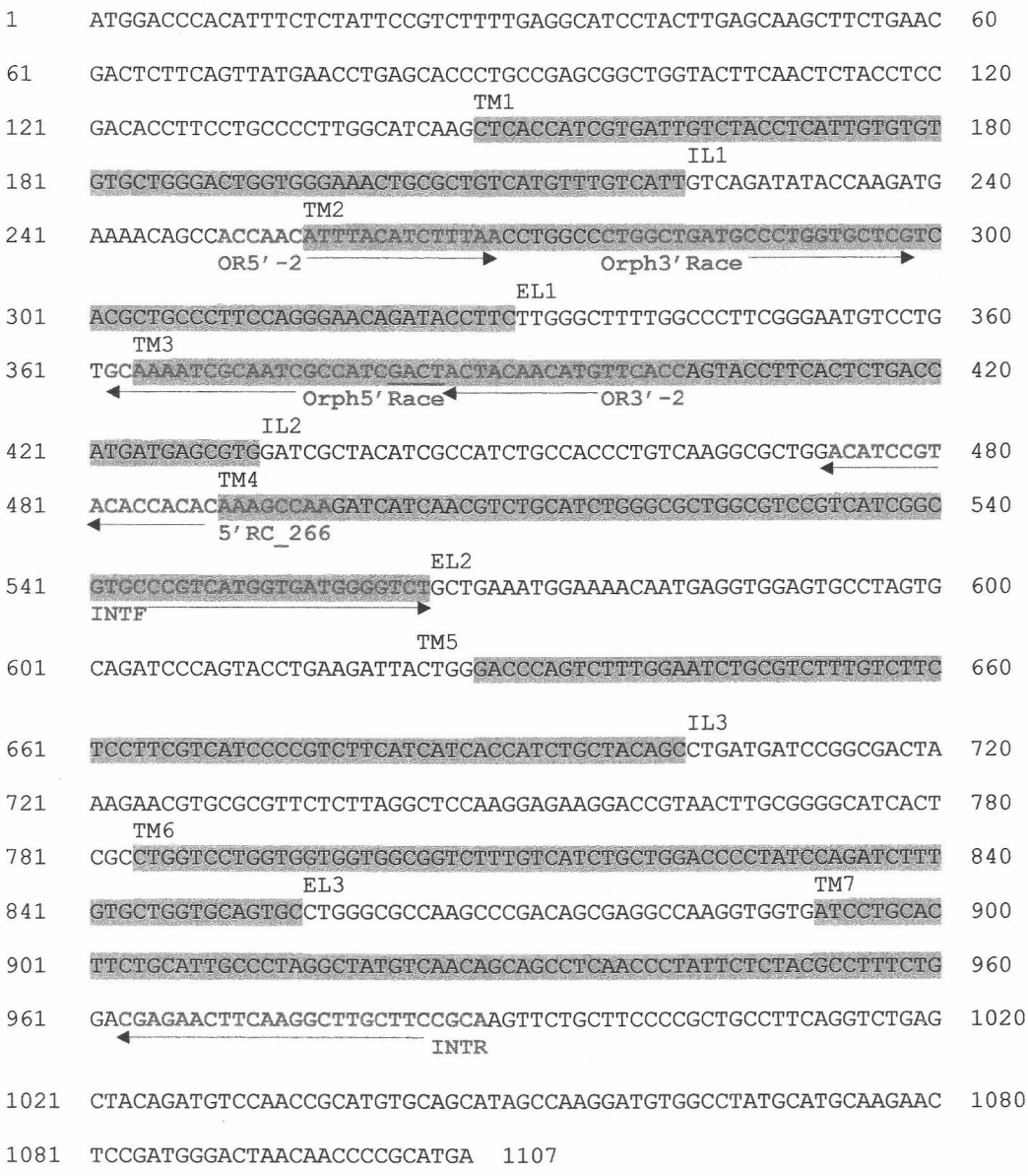

Figure 2.1: A map of the primers used in the isolation of the nORL receptor cDNA sequence is depicted. Primer names and sequences are in bold, with arrows depicting primer directionality. Underlined nucleotides indicate regions of primer overlap. Transmembrane domains (TM) are highlighted in gray. IL and EL refer to intracellular and extracellular loops, respectively. Note: the degenerate sequences for OR5'-2 and OR3'-2 are shown in Table 2.1; this map depicts the primer locale and newt-specific ORL nucleotide sequences.

RESULTS

The coding region for the cDNA cloned from newt brain and its deduced amino acid sequence are shown in Figure 2.2. The deduced amino acid sequence is 368 amino acids long and contains seven hydrophobic regions that share identity with the transmembrane domains (TMD) of opioid G-protein coupled receptors (GPCR) (Figure 2.3 & Table 2.2). The sequence also shares identity with opioid receptors in regions considered to be extracellular and intracellular loops (Figure 2.3 & Table 2.2). The deduced amino acid sequence contains a number of putative consensus sites for post-translational modifications common to other characterized opioid receptors and GPCRs in general. These include three asparagine residues within the N-terminal domain, two cysteine residues in the intracellular C-terminal domain, and a cysteine residue in extracellular loops one and two (Figure 2.2).

The deduced amino acid sequence of the newt cDNA has the highest identity (45-57%) to GPCRs in the opioid receptor family (Table 2.3). When compared to the amino acid sequences of the human opioid receptor types (μ, δ, κ, ORL1), the newt receptor has 57% identity to the ORL1 receptor, and 51%, 49%, and 48% identity to the κ-, μ-, and δ- opioid receptor types, respectively (Table 2.3). When the deduced amino acid sequences of the newt, human ORL1 and κ receptors are aligned, the newt receptor appears to be a composite of the ORL1 and κ receptor sequences (Figure 2.4). The relatedness of the newt receptor sequence to other opioid receptor sequences from various species is illustrated in the dendrogram in Figure 2.5. From the dendrogram it can be seen that the newt receptor falls between the κ– and ORL1 receptor types in terms of degree of sequence identity, but overall shares more identity with the ORL1 receptor.

When compared across all the human opioid receptor types, the regions with the highest amount of amino acid conservation are found in the TMDs and the intracellular loops (48% and 68%, respectively) (Figure 2.3 & Table 2.2). A comparison of the newt receptor to each human receptor type reveals that the

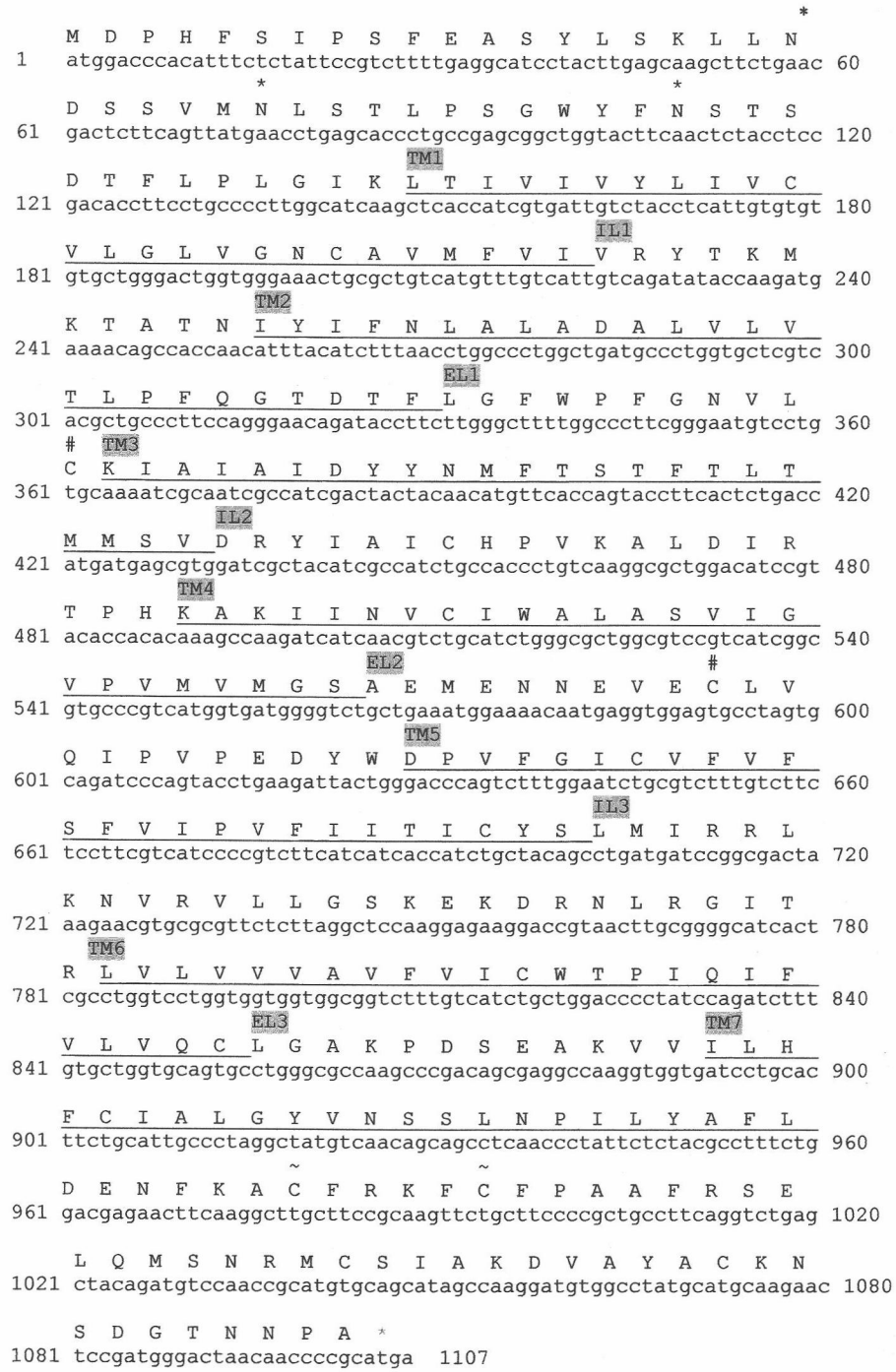

Figure 2.2: The cDNA nucleotide sequence for the coding region of the nORL receptor is shown aligned with its deduced amino acid sequence. Underlined regions = putative transmembrane domains (TM). IL = intracellular cytoplasmic loop; EL = extracellular loop. * = potential glycosylation sites, # = disulfide bridge formation, ~ = potential palmitoylation sites.

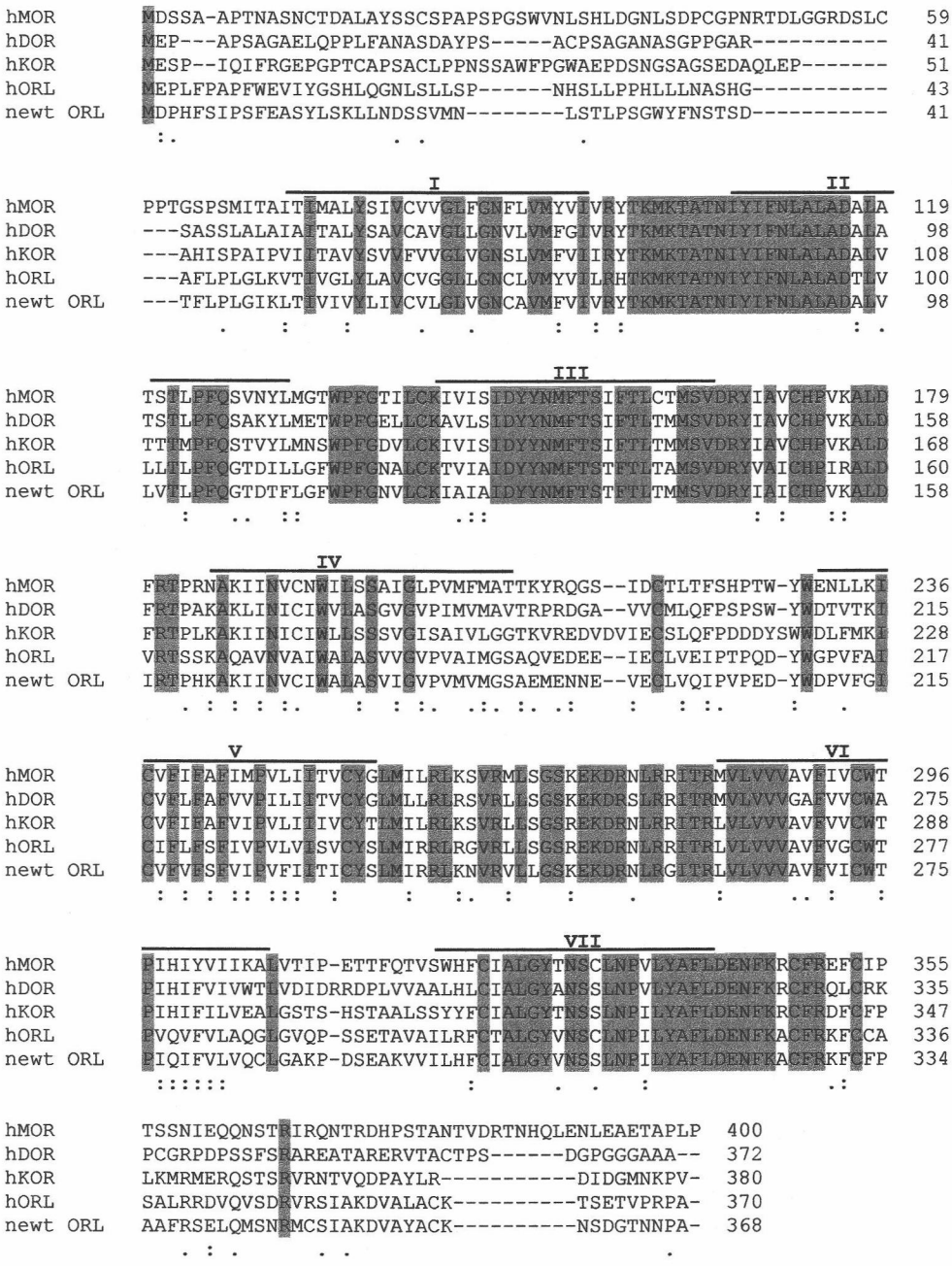

Figure 2.3: The deduced amino acid sequence for the nORL receptor is shown aligned with the human μ, δ, κ, and ORL1 opioid receptors (hMOR, hDOR, hKOR, hORL). Putative transmembrane domains are indicated by a solid line above the sequence. Gray highlighting indicates a single, fully conserved residue across all the receptors. Conservation of strong or weak groups is indicated by a (:) or (.) below the residue, respectively.

Table 2.2: Sequence identity for receptor domains of the nORL receptor and the human ORL1, κ, δ, and μ opioid receptors (hORL, hKOR, hDOR, hMOR). Transmembrane domain = TMD, extracellular loop = EL, and intracellular loop = IL. (*) EL2 of the hKOR has 3 insertions for a total of 24 residues; (**) EL3 of the hDOR has one insertion for a total of 12 residues.

Structural Elements	Conserved/Total Amino Acid Residues(%)				
	All Subtypes	hORL	hKOR	hMOR	hDOR
TMD I	10/25 (40)	17/25 (68)	15/25 (60)	15/25 (60)	12/25 (48)
TMD II	15/25 (60)	22/25 (88)	18/25 (72)	17/25 (68)	17/25 (68)
TMD III	17/24 (71)	21/24 (88)	21/24 (88)	19/24 (79)	19/24 (79)
TMD IV	6/25 (24)	18/25 (72)	14/25 (56)	16/25 (64)	17/25 (68)
TMD V	9/25 (36)	15/25 (60)	15/25 (60)	13/25 (52)	15/25 (60)
TMD VI	10/25 (40)	20/25 (80)	20/25 (80)	16/25 (64)	16/25 (64)
TMD VII	15/23 (65)	20/23 (87)	19/23 (83)	18/23 (78)	19/23 (83)
TMD I-VII	82/172 (48)	133/172 (77)	122/172 (71)	114/172 (66)	115/172 (67)
EL 1	6/11 (55)	8/11 (73)	6/11 (55)	6/11 (55)	5/11 (45)
EL 2	2/21* (10)	13/21 (62)	7/21 (33)	4/21 (19)	7/21 (33)
EL 3	0/11** (0)	5/11 (45)	3/11 (27)	2/11 (18)	1/11 (9)
EL 1-3	8/43 (19)	26/43 (60)	16/43 (37)	12/43 (28)	13/43 (30)
IL 1	9/11 (82)	9/11 (82)	10/11 (91)	11/11 (100)	11/11 (100)
IL 2	12/19 (63)	13/19 (68)	16/19 (84)	16/19 (84)	16/19 (84)
IL 3	18/27 (67)	21/27 (78)	21/27 (78)	22/27 (81)	19/27 (70)
IL 1-3	39/57 (68)	53/47 (75)	47/57 (82)	49/57 (86)	46/57 (81)

Table 2.3: Amino acid sequence identity between the nORL receptor and the μ, δ, κ and ORL1 opioid receptors from other species.

Receptor Type	Species	Identity (aa)
ORL1	Human	57%
	Mouse	56%
	Rat	55%
	Pig	56%
	Guinea Pig	55%
	Zebrafish	49%
κ	Human	51%
	Mouse	51%
	Rat	50%
	Guinea Pig	51%
μ	Human	49%
	Mouse	49%
	Rat	49%
	Pig	46%
	Cow	49%
	Zebrafish	49%
	Macaque	50%
	Whitesucker	47%
δ	Human	48%
	Mouse	45%
	Rat	45%
	Zebrafish	47%

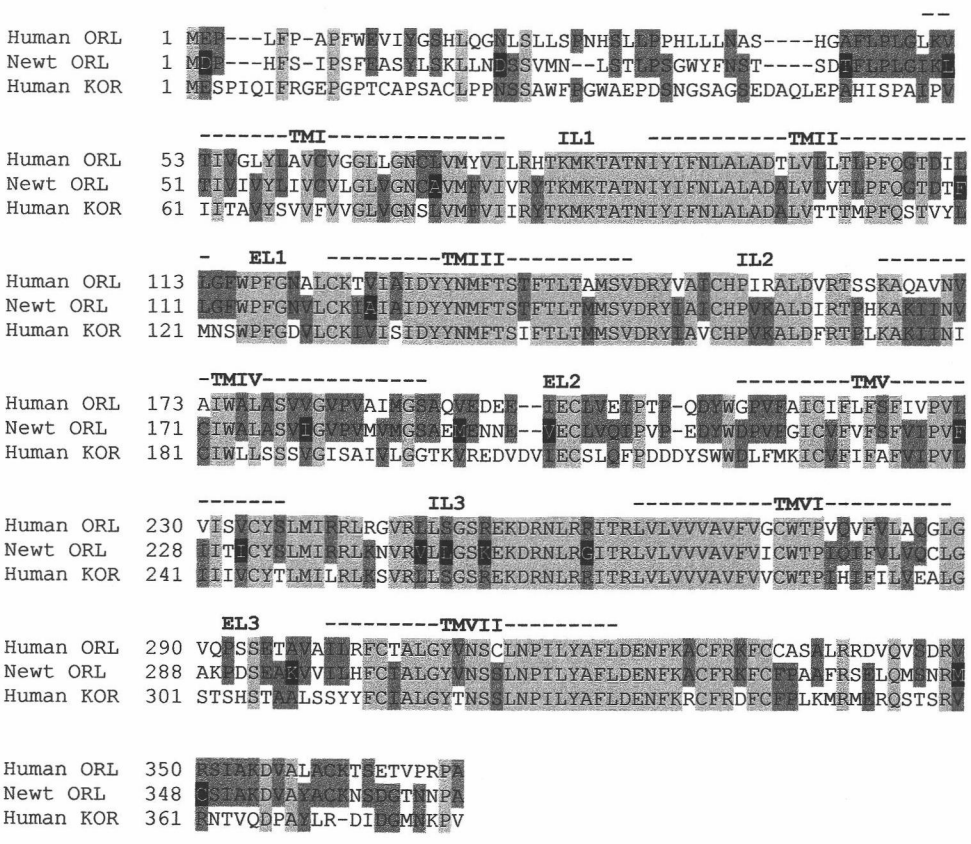

Figure 2.4: Alignment of the deduced amino acid sequences for the human ORL1 receptor, nORL receptor, and human κ opioid receptor (KOR). Putative transmembrane domains (TM) are depicted by dashed lines. IL and EL refer to intracellular loop and extracellular loop, respectively. Residues which are fully conserved across the three receptors are highlighted in light gray. Identical residues shared by two of the receptors are highlighted in dark gray. Residues, which are unique to the newt but are shared between the human ORL1 and KOR, are highlighted in black. A white background indicates no consensus between sequences.

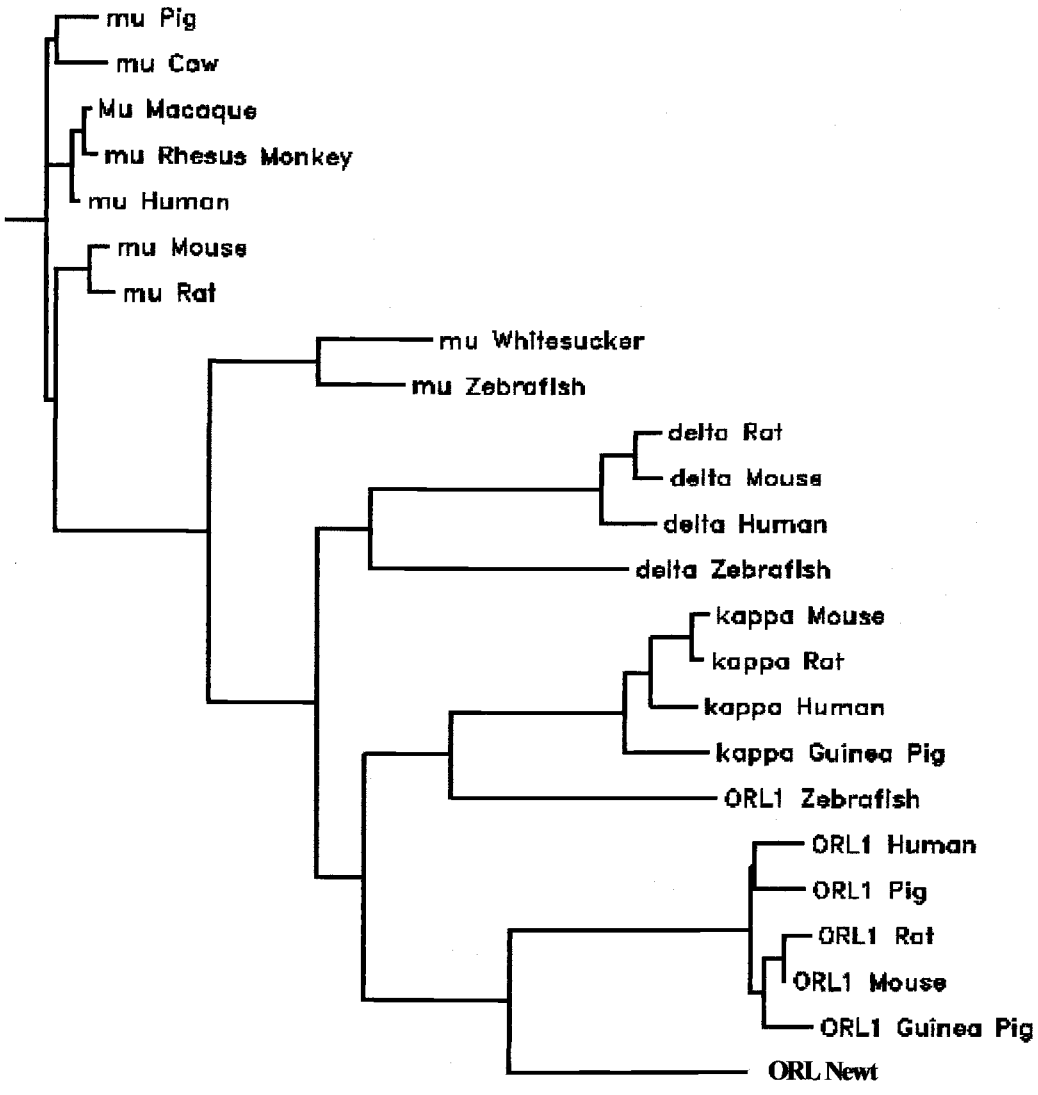

Figure 2.5: A dendrogram depicting the relatedness of the nORL receptor to known opioid receptor types from other species. The species and opioid receptor type (mu, delta, kappa, ORL1) are listed.

degree of similarity in the TMDs is fairly equal between types (66-77%) (Table 2.2). However, when the identity in the extracellular loops is compared, the newt receptor shares 60% identity with the human ORL1, as opposed to only 28-37% with the other receptor types (δ, μ, κ) (Table 2.2). Furthermore, the second extracellular loop (EL2) of the newt receptor contains a high number of acidic residues, a characteristic shared only by the ORL1 and κ receptor types (Figure 2.4).

The newt receptor shares a number of residues with the mammalian ORL1 receptor that have been implicated in ligand binding and receptor functionality by previous studies (Figure 2.3). Among these are Glu^{286}, Asp^{130} and Tyr^{131} in TMIII, Phe^{220} and Phe^{224} in TMV, and Trp^{276} in TMVI (Mouledous et al. 2000). Also, the newt receptor shares identity with the classic opioid receptors at three specific residues (I^{303}, I^{277}, I^{279}) that are not conserved in the mammalian ORL1 receptor, which may enable the newt receptor to recognize κ ligands (Meng et al. 1996a; Meng et al. 1998) (Table 2.4 and Figure 2.3).

Table 2.4: Mutations in a study by Meng et al. (1996a) shown to increase the affinity of the rat ORL1 for κ-selective ligands. The wild-type rat ORL1 (wt rORL) sequence is depicted along with the mutant rat ORL1 (mut ORL) sequence and the deduced nORL receptor sequence. Residues highlighted gray indicate the amino acid(s) targeted for mutation, the mutant residue, and the residue found in the nORL receptor at that position for comparison. When a construct was created that contained both the TMVII and TMVI amino acid substitutions (a total of 4 residues), the resultant receptor was able to bind both κ and ORL1 ligands with nanomolar affinity.

Receptor	TMV	TMVII	TMVI
wt rORL	VFAIC	FCTAL	PVQVF
mut ORL	VFKIC	FCIAL	PIHIF
newt ORL	VFGIC	FCIAL	PIQIF

DISCUSSION

Using the roughskin newt, *Taricha granulosa*, a cDNA was obtained that has the greatest sequence identity to the mammalian opioid receptor-like (ORL1) receptor, also termed the orphanin FQ or nociceptin receptor. In addition, the newt receptor cDNA has identity with the κ opioid receptor (KOR) at specific residues that predict its ability to bind both ORL1 and κ ligands with high affinity. This sequence information provides insights into the evolution of the opioid receptors in vertebrates. To the best of our knowledge, this is the first complete opioid receptor cDNA sequence obtained from an amphibian.

The newt sequence (Figure 2.2) shares a number of highly conserved characteristics with receptors belonging to the G-protein coupled receptor (GPCR) superfamily, supporting the conclusion that it codes for a GPCR. The deduced amino acid sequence contains seven hydrophobic regions that have sequence identity to GPCR membrane-spanning domains (Law et al. 1999; Minami & Satoh, 1995). The newt sequence also contains the following GPCR characteristics: asparagine residues within the N-terminal domain that function as potential sites for N-linked glycosylation (Minami & Satoh, 1995), cysteine residues in the intracellular C-terminal domain that are potential palmitoylation sites (Ovchinnikov et al. 1988; O'Dowd et al. 1989), and two cysteine residues, located in extracellular loops one and two, that may connect the loops by forming a disulfide bridge (Dixon et al. 1987; Karnik et al. 1988). In addition, there are a number of serine and threonine residues in the putative cytoplasmic regions, presenting potential sites for phosphorylation by protein kinases. The phosphorylation of homologous sites in GPCRs is a process thought to affect receptor regulation—namely desensitization (Lefkowitz et al. 1998; Law et al. 2000).

Sequence identity between the newt cDNA and other types of GPCRs indicates it codes for an opioid receptor. Overall, the newt receptor shares ~50% amino acid sequence identity with the four mammalian opioid receptor types (μ, δ, κ, ORL1),

and has the highest identity to the human ORL1 (57%) (Table 2.3). A comparison of specific receptor regions across receptor types reveals that the extracellular loops share the least identity, perhaps to preserve their ability to recognize and discriminate between similar ligands (Wang et al. 1994b, 1995; Meng et al. 1996b; Mollereau et al. 1999). In these regions, the identity of the newt receptor to the human ORL1 is 60%, compared to only 28-37% with the other receptor types (Table 2.2). Based on these observations, we conclude the newt cDNA codes for an ORL1-like opioid receptor (referred to hereafter as nORL).

The transmembrane domains (TMD) of opioid receptors form an'opioid binding pocket', a region where specific ligand-receptor interactions cause receptor activation. The conservation of identity in these regions (66-77%) suggests the nORL receptor possesses a transmembrane binding pocket similar to the alkaloid binding pocket of the opioid receptors. In particular, five residues within the opioid binding pocket of the mammalian ORL1 are known to greatly influence ligand affinity: Asp^{130} and Tyr^{131} in TMD3, Phe^{220} and Phe^{224} in TMD5, and Trp^{276} in TMD6 (Mouledous et al. 2000). Mutation to alanine of each of the aforementioned residues has an adverse effect on receptor affinity and reactivity towards orphanin FQ (oFQ), the endogenous ligand of the ORL1 (Mouledous et al. 2000). The nORL receptor has the mammalian ORL1 receptor residues at these five sites, indicating their functional importance may be similar in an amphibian. In addition, a residue located at the end of TMD6 (Glu^{286}), which plays a pivotal role in the transduction of signal in the human ORL1 (Mouledous et al. 2000), is also conserved in the nORL receptor. Therefore, based on sequence analysis, it appears the nORL receptor shares a number of key residues involved in ligand affinity and receptor activation with its mammalian counterpart.

Although the newt receptor has the highest sequence identity to the mammalian ORL1, it shares a number of residues with the κ opioid receptor (KOR) that are implicated in κ ligand recognition. Meng et al. (1996a) showed that the rat ORL1 receptor could recognize several dynorphin-derived peptides by mutating as few as

four ORL1 residues to the conserved opioid receptor counterparts ($VQV^{279-281}$ mutated to IHI in TMD6, and T^{305} mutated to I in TMD7). These amino acid substitutions created a mutant receptor capable of binding both orphanin FQ and prodynorphin products with subnanomolar affinity. The addition of a fifth mutation to the rat ORL1 receptor construct (A^{216} mutated to K in TMD5) caused its affinity towards selective opioid antagonists to increase 2-3 orders of magnitude (Meng et al. 1998). Of these five residues the newt receptor shares three with the classical opioid receptors, one with the ORL1, and has one residue unique to the nORL (Table 2.4). In addition, the alignment of the nORL receptor with the mammalian ORL1 and KOR sequences demonstrates that it is a partial hybrid of the two receptor types (Figure 2.4), alternating between KOR- and ORL1-specific residues throughout the sequence. Therefore, the pharmacology of the nORL receptor may be substantially different from the mammalian ORL1, perhaps displaying affinity for opioid alkaloids and κ selective peptides. In this respect the nORL receptor may be a structural and/or functional intermediate between the κ– and ORL1 receptor types.

It has been hypothesized that the four opioid precursor genes (proenkephalin, POMC, prodynorphin, and proorphanin) arose by sequential duplication of a common ancestral gene over the past five hundred million years, and the emergence of the cognate receptor proteins (δ, μ, κ, ORL1) paralleled the divergence of the peptide genes (Douglass et al. 1984). Recently, the first complete non-mammalian vertebrate proorphanin cDNA gene sequence was obtained from the sturgeon, *Acipencer transmontanus* (Danielson et al. 2001). The sturgeon oFQ peptide retains the classical opioid peptide core sequence (YGGF), whereas all mammalian oFQ peptides have a modified opioid-like core (FGGF). The sturgeon oFQ, with the N-terminal tyrosine, shows affinity for the mammalian ORL1 as well as the μ–, δ–, and κ– opioid receptors. The authors suggest that the sturgeon oFQ represents an intermediate stage in the evolution of the proorphanin gene, and a "transitional state in the eventual functional isolation of the orphanin

heptadecapeptide from the classical opioids" (Danielson et al. 2000). If this is true, then receptor coevolution may have produced cognate receptor intermediates in some species capable of recognizing orphanin FQ and the classical opioid peptides. Perhaps the nORL receptor is an evolutionary intermediate between the κ- and ORL1 receptor types.

The complete cDNA sequences for all four opioid precursor genes and their cognate receptors have only been obtained from mammals (Genbank). To date, full-length cDNA sequences for the POMC and proenkephalin genes, plus a partial cDNA sequence for the prodynorphin gene, have been obtained from amphibians (Genbank). However, no amphibian proorphanin sequences have been obtained. In parallel with these findings, although no complete opioid receptor cDNA sequences have been reported for amphibians, partial sequences for the δ–, μ–, and κ–like receptors have been obtained from bullfrog (*Rana catesbeiana)* genomic DNA (Li et al. 1996). Based on these findings, it appears that *at least* three opioid peptide and receptor types will be present in amphibians—prodynorphin, proenkephalin, and POMC, and κ–, δ–, and μ–like receptors, respectively. This prediction is supported by complete and partial cDNA sequences obtained for μ-, δ-, κ-, and ORL1- like receptors from a 'lower' vertebrate, the white sucker *Catostomus commersoni*, indicating multiple opioid receptor types arose early in evolution (Darlison et al. 1997).

The presence of multiple opioid peptide and receptor types in amphibians is also supported by previous behavioral and pharmacological studies. Selective opioid agonists and antagonists have differential influences on nociceptive (Stevens & Pezalla, 1983, 1984; Pezalla 1983; Pezalla & Dicig, 1984; Stevens et al. 1987; Stevens & Rothe, 1997), locomotor (Pezalla, 1983; Deviche et al. 1989; Pezalla & Stevens, 1984; Lowry et al. 1990), and sex behaviors (Deviche & Moore, 1987) in amphibians, indicating a separation of function between receptor types. In accord with this, multiple opioid-like receptor types (μ, δ, κ) and subtypes (κ1, κ2) have been characterized in amphibians with binding studies using brain tissue from toads

(Ruegg et al. 1980, 1981; Simon et al. 1982) and frogs (Simon et al. 1984, 1985, 1987; Benyhe et al. 1990, 1994; Borsodi et al. 1986; Mollereau et al. 1988; Makimura et al. 1988; Wollemann et al. 1994). The binding profiles and relative abundance of these sites differ from their mammalian counterparts. The κ-like site appears to be the most prevalent binding site, and these sites display a greater affinity for μ- and δ- selective opioids, and a lesser affinity for κ-selective opioids, *compared* to the mammalian κ site (Mollereau et al. 1988; Simon et al. 1982). Also, species differences in the stereoselectivities of opioid binding sites for specific ligands have been reported (Benyhe et al. 1992). In light of these studies, we would expect to find multiple types of opioid receptors in amphibians that may differ structurally and functionally from their mammalian homologues. To date, no studies have been done on amphibians to characterize an oFQ binding site.

The results of the present study indicate that an opioid-like receptor cDNA with high sequence identity to the mammalian ORL1 receptor, as well as numerous residues specific to the mammalian KOR, is present in an amphibian. The sequence information indicates that the newt receptor may represent an evolutionary intermediate of the κ- and ORL1- receptors. The expression of the full-length nORL receptor in a tissue culture system will allow its pharmacological characterization, and will determine if the expressed receptor is capable of recognizing both oFQ and prodynorphin peptides as predicted by the sequence analysis. Future studies on the expressed receptor will also investigate its coupling to second messengers and its role in cellular physiology. Cloning additional full-length opioid receptors from amphibians will provide information on the evolution of the functional opioid system, and contribute to the current body of knowledge regarding this important neuropeptide system.

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CHAPTER 3:

THE TISSUE DISTRIBUTION AND REGULATION OF THE NEWT ORL1-LIKE OPIOID RECEPTOR

ABSTRACT

In mammals, orphanin FQ (oFQ), also known as nociceptin, is the endogenous ligand for the opioid receptor-like (ORL1) receptor, a G protein coupled receptor that shares a high degree of sequence identity with the opioid receptors. The oFQ/ORL1 system is implicated in a broad array of physiological effects, including responses to stress. Recently, we obtained a complete cDNA sequence that encodes an opioid-like receptor from the roughskin newt, *Taricha granulosa*, and has high sequence identity to the mammalian ORL1 receptor. It is referred to hereafter as the newt ORL1-like (nORL) receptor. In the first experiment of the present study, reverse-transcriptase PCR was used to determine the tissue distribution of the nORL receptor. Using this methodology, the nORL receptor was detected in the brain, spinal cord, and lungs of the roughskin newt. The nORL receptor was not detected in spleen, small intestine, heart, liver, sperm duct, bladder, or kidney. In the second experiment of the current study, we quantified the nORL receptor mRNA at the level of the spinal cord following chronic exposure to the stress hormone corticosterone (CORT). Animals were exposed to CORT for 10 days by surgically implanting CORT-filled Silastic capsules. Real-time PCR was used to quantify nORL receptor mRNA levels in treated and control animals. Samples from the same animals were used to simultaneously quantify mRNA levels for a known CORT receptor, the intracellular glucocorticoid receptor (iGR). Our results indicate that chronic exposure to CORT did not significantly affect the level of mRNA expression for either the nORL receptor or the iGR, as compared to control animals receiving no hormone treatment. These studies indicate that the nORL receptor is primarily located in the central nervous system, and its transcriptional regulation is not affected by chronic exposure to CORT at the level of the spinal cord.

INTRODUCTION

The opioid receptor-like (ORL1) receptor is a G-protein coupled receptor (GPCR) that shares high sequence identity with the three recognized opioid receptor types, mu (μ), delta (δ), and kappa (κ) (Mollereau et al. 1994). The endogenous peptide for the ORL1 receptor, known as orphanin FQ (oFQ) (Reinscheid et al. 1995) or nociceptin (Meunier et al. 1995), is a heptadecapeptide whose N-terminal tetrapeptide sequence is related to that of the opioid peptides (FGGF vs YGGF), and whose basic core is similar to dynorphin, the endogenous peptide for the κ receptor. In spite of these similarities, the ORL1 receptor does not recognize the majority of opioid ligands, and oFQ has low affinity for the classic opioid receptors (Meng et al. 1996; Reinscheid et al. 1995). At the cellular level the ORL1 receptor couples to the same G_i/G_o protein-mediated second messenger systems as the opioid receptors: inhibition of adenylate cyclase, activation of an inwardly rectifying K^+ conductance, and inhibition of voltage-sensitive Ca^{2+} channels (for review, see Meunier et al. 1997). These intracellular effectors generally act to inhibit cellular excitability, and suggest a role for the oFQ/ORL1 system in the modulation of neuronal activity.

In order to understand the physiological/behavioral phenomena of a given neuropeptide, the distribution of the peptide and its receptor must be elucidated. Distribution studies on the ORL1 receptor have shown it to be located extensively throughout the brain and spinal cord, as well as in select tissues of the periphery (for reviews see Mollereau et al. 2000; Darland et al. 1998; Meunier, 1997; and Henderson & McKnight, 1997). The broad distribution of the ORL1 receptor in the central nervous system (CNS) mirrors its involvement in a range of physiological effects. oFQ increases or decreases nociception depending on the site of administration and dose (Reinscheid et al. 1995; Mogil et al. 1996a, 1996b; Tian et al. 1997; Rossi et al. 1996), increases food intake in satiated rats (Pomonis et al.

1996), increases or decreases locomotion (Reinscheid et al. 1995; Florin et al. 1996), and impairs spatial learning (Sandin et al. 1997).

The ORL1 receptor is located in brain regions involved in the integration of stressful stimuli, including the locus coeruleus, central gray, raphe nucleus, hypothalamus, and amygdala (see Mollereau et al. 2000), suggesting that this system may be involved in the control of behavioral responses to stress. Support for this role has come from studies demonstrating that oFQ acts as an anxiolytic when administered intracerebroventricularly (i.c.v.) to rats and mice (Jenck et al. 1997). In addition, mice lacking the oFQ gene have higher basal plasma corticosterone levels than control mice, and a reduced ability to adapt to repeated stressors (Koster et al. 1999). Based on these studies, it has been hypothesized that exposure to stress causes a release of oFQ, which acts to inhibit excessive anxiogenic responses (Walker & Koob, 1997).

A hormone's activity at any given target is dependent on the presence of specific receptors at the target, the receptor density, the receptor's sensitivity, and other factors. To turn off the cellular response to a hormone, the receptors must be desensitized (rendered incapable of transducing signal) and/or removed from the membrane (downregulated). Opioid receptor desensitization and downregulation following chronic exposure to opiates has been well documented (Borgland, 2001). In addition to receptor downregulation, a decrease in the expression of the receptor may regulate ligand activity at a cell. In the case of the opioid receptors, receptor messenger RNA (mRNA) levels have been shown to be both up-and down-regulated (i.e. Yoshikawa et al. 2001; Kim et al. 1995) in response to chronic exposure to agonists, as well as exhibiting no change (i.e. Castelli et al. 1997). Also, the reports of unchanged or elevated receptor mRNA levels following chronic exposure to an opioid agonist outnumber those reporting a decrease in mRNA expression, even though there is often an observable decrease in receptor protein densities in these cases (see Law et al. 2000). Hence, no consensus has

been reached on the regulation of opioid receptor mRNA expression following chronic exposure to opioid drugs.

As mentioned previously, it is hypothesized that oFQ is released following exposure to stress, and inhibits excessive anxiogenic responses (Walker and Koob, 1997). If this hypothesis is true, an increase in oFQ peptide and/or mRNA would be expected to occur in response to stress. Fluctuating oFQ peptide levels may initiate changes in ORL1 receptor density and sensitivity at target sites. To date, no studies have been conducted to examine the regulation of the oFQ peptide or the ORL1 receptor following exposure to acute or chronic stress.

Recently, we obtained a complete cDNA encoding an ORL1-like opioid receptor from an amphibian (*Taricha granulosa*). In the current study, experiments were conducted to address the transcriptional regulation of the newt ORL1-like (nORL) receptor following chronic exposure to stress. In study A, potential sites of receptor regulation were established by determining the tissue distribution of the nORL receptor using reverse-transcriptase PCR. In study B, the mRNA expression of the nORL receptor in the spinal cord following chronic exposure (10 days) to the stress hormone corticosterone (CORT) was quantitated using real-time PCR. The quantification of mRNA for a known CORT receptor, the intracellular glucocorticoid receptor (iGR), was conducted simultaneously. These data provide new information on the distribution of an ORL1-like receptor in an amphibian, and on the regulation of both a GPCR and an intracellular receptor following chronic exposure to a glucocorticoid involved in the stress response.

MATERIALS AND METHODS

Study A) tissue distribution of the nORL receptor by RT-PCR

RNA purification and reverse transcription

Six roughskin newts (*Taricha granulosa*) were anesthetized on ice and rapidly decapitated. The newts were then dissected, and the following organs were collected and immediately frozen in liquid nitrogen: brain, spinal cord, spleen, small intestine, heart, lung, liver, sperm duct, bladder, and kidney. The tissues were stored at –80°C until the RNA isolation was started. RNA was purified from collected tissues using the reagents and protocol provided with RNA STAT-60 (Tel-Test, Inc. Friendswood, TX). Purified RNA was resuspended in RNase-free H_2O, and quantified in a Beckman DU530 spectrophotometer (Beckman Coulter, Inc.) Purified RNA was also examined by 1.5% agarose gel electrophoresis to check for the presence of genomic DNA, as well as to confirm the presence of the expected ribosomal bands (4.7 kb and 1.9 kb).

The Gibco Superscript System for RT-PCR First-Strand Synthesis and protocol were used to reverse-transcribe the purified RNA into cDNA using an oligo-dT primer (Invitrogen, Carlsbad, CA). Up to 5 μg of total RNA from each tissue was used per reverse-transcription reaction. The resultant cDNA was stored at –20°C.

Primer design and PCR

Newt ORL1-specific primers were designed based upon the cloned newt ORL1 (nORL) receptor nucleotide sequence (see chapter 2). Primers were designed for regions known to be highly specific to the nORL in an attempt to prevent the possibility of cross-reactivity with another similar receptor. Extracellular loop 2 of

the ORL1 (newt and mammalian) is known to be one of the most unique regions of the receptor; therefore, the sense primer (DISTF) corresponds to amino acid residues 184-190 in this region (newt ORL1 numbering). The antisense primer (DISTR) corresponds to amino acid residues 278-284 at the end of transmembrane domain VI. The primer names and nucleotide sequences are as follows:
DISTF: 5'-GTGATGGGGTCTGCTGAAAT-3' (n.t. 553-572)
DISTR: 5'-CACTGCACCAGCACAAAGAT-3' (n.t. 835-854)

Specificity of the nORL primers was demonstrated in PCR reactions using a positive control template (nORL cDNA), a no-template control (H_2O), and a reaction containing the cDNA for the rat kappa opioid receptor (rKO1R) as template (provided by Dr. Huda Akil). The nORL primers successfully amplified the nORL template, but failed to amplify the rKO1R template, indicating the primers did not cross-react with the kappa opioid receptor cDNA (data not shown). In addition, no products were produced in the no-template (H_20) control reaction.

The distribution of the nORL receptor was determined by semi-quantitative PCR. 20 µl reactions were set up with the specific primers (0.5 µM) and the cDNA (10-150 ng) from the collected tissues. The quality and quantity of the cDNA was controlled for in PCR reactions conducted simultaneously using β-actin primers (designed by Dr. Joanne Chu). All PCR reactions contained 2.0 µl of 10x PCR Buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, and 0.4 U Taq polymerase (MBI Fermentas, Hanover, MD). The following cycle conditions were used: 94°C for 2 min; 35 cycles of 94°C for 10 s, 60°C for 30 s, 72°C for 1 min; 72°C for 5 min; and hold at 4°C. The PCR reactions were examined by 1.5% agarose gel electrophoresis and ethidium bromide staining for the nORL-specific amplicon (303 bp) or the actin-specific amplicon (150 bp).

Study B) nORL receptor and iGR mRNA expression in the newt spinal cord in response to chronic exposure to corticosterone

Hormone treatment

Thirty-six male newts were collected from the Corvallis watershed (Benton County, OR) and subsequently maintained in dechlorinated water tanks on a 12 hour light:12 hour dark cycle, at approximately 14°C. The animals were fed earthworms every 2-3 days.

Two weeks after collection, the newts were randomly assigned to one of three treatment groups: chronic exposure to corticosterone, sham-operated control, or no-treatment control. For delivery of the hormone, 0.75 cm lengths of Silastic tubing were plugged at one end with 0.25 cm of silicon and then packed with corticosterone powder (~9 mg/capsule, Sigma-Aldrich, USA). Sham-capsules were prepared the same way but left empty. The capsules were soaked in Ringer's solution for 48 hours before use.

Capsules (corticosterone or sham) were placed below the peritoneal lining of anesthetized animals (0.2% MeSH, 10 min) through a small ventral incision above the left hindlimb. The incision was rapidly sealed with Med Bond Veterinary Glue and the animals were placed in water-filled buckets for recovery, and subsequently returned to separate holding tanks as designated by treatment group.

Ten days following surgery animals were sacrificed as described in Study A, and brain, spinal cord, and plasma samples were collected from each newt. The brain and spinal cords were immediately flash-frozen in liquid nitrogen, and stored at –80°C until further use. Plasma was separated (10,000 rpm for 10 min at 4°C) from whole blood in heparinized (0.25 g/ml) tubes and stored at –80°C until being sent to the Oregon Regional Primate Center for analysis of corticosterone levels by radioimmunoassay.

RNA isolation and purification, and cDNA synthesis for real-time PCR

Total RNA was isolated from the brain and spinal cord samples using the RNAqueous-4PCR kit and protocol from Ambion (Austin, TX). Briefly, the kit's methodology is based on the initial disruption of tissue in a solution containing guanidinium thiocyanate, a strong denaturant that lyses cell membranes and rapidly inactivates cellular ribonucleases. Silica-based filters are used for the isolation of purified RNA from the cell lysis. Problems arose at the filter step while isolating RNA from the brain samples using this kit. Because of frequent filter clogging, perhaps due to an inadequate volume of lysis solution during the initial tissue disruption, the resultant eluate produced extremely low quantities of brain RNA with unacceptable 280:260 OD ratios. Therefore, the brain samples were eliminated from the remainder of the study. However, the quantity and quality of the spinal cord RNA samples were adequate for cDNA synthesis (280:260 OD of 1.8-2.0). cDNA was generated by RT-PCR with random hexamer primers (Fermentas First Strand cDNA Synthesis kit, MBI Fermentas, Hanover, MD) and stored at $-20^{\circ}C$.

Primer design for real-time PCR

New primers were specifically designed for use in the real-time PCR reactions using Primer Express software (Perkin-Elmer Applied Biosystems, Foster City, CA). Based on a number of candidate forward and reverse primers selected by the software, three primer pairs were chosen for the nORL receptor and the intracellular glucocorticoid receptor (iGR) (see Table 3.1 for primer names and sequences). The iGR primers were designed based on a partial cDNA sequence for the newt iGR (predicted to correspond to amino acids 134-244 of the *Xenopus laevis* full-length iGR) previously obtained by Carla Richardson (Figure 3.1). β-

actin primers were previously designed for use in endogenous control reactions (Dr. Joanne Chu). All primers were synthesized by One Trick Pony (Ransom Hill Bioscience, Inc. Ramona, CA), and diluted to 50 μM in ddH_2O upon arrival.

Table 3.1: The names and nucleotide sequences of the primers designed for quantitative real-time PCR are shown. The amino acid numbering is based on the nORL numbering in the case of the nORL primers, and the predicted corresponding full-length *X. laevis* residue numbering in the case of the iGR primers.

Primer Name	Primer Sequence (5'→3')	Amino Acid Residues	Amplicon
ORLQRT1-F ORLQRT1-R	CTGGTGCTCGTCACGCTG CGCTCATCATGGTCAGAGTGAA	97-102 137-143	142 bp
ORLQRT2-F ORLQRT2-R	AATGAGGTGGAGTGCCTAGTGC GCCGGATCATCAGGCTGTAG	194-201 233-238	136 bp
ORLQRT3-F ORLQRT3-R	TCTTCATCATCACCATCTGCTACAG GTGATGCGCCGCAAGTTAC	227-234 255-260	103 bp
iGRQRT1-F iGRQRT1-R	TACTCCTGGATGTTCCTGATGGT GCATACATTCGATCCTTATTGATGA	146-153 177-184	116 bp
iGRQRT2-F iGRQRT2-R	TCTTGGATGGAGATCTTACAAGCA TGCATACATTCGATCCTTATTGATG	156-163 177-184	88 bp
iGRQRT4-F iGRQRT4-R	GCACGCCTTCAAGTGTCGTA CCTTAGGAATTGTACAGAGGAGCAT	202-208 218-226	73 bp

Real-time primers were tested for specificity in PCR reactions using 100 ng of newt brain cDNA, and then optimized (SYBR Green PCR Master Mix Protocol (pgs 5.2-5.8), ABI website) before use in the real-time PCR reactions on the experimental samples. One primer pair for each gene of interest (ORLQRT2 & iGRQRT4) was selected for the experimental real-time assays based on the primer's specificity and efficiency.

1 AATTTGCACTTTGACGACCAGATGACTCTTCTCCAGTACTCCTGGATGTTCCTGATGGTT

61 TTCGGTCTTGGATGGAGATCTTACAAGCAAGCAAATGGAATCATGTTGTGCTTCGCCCCG

121 GACTTGATCATCAATAAGGATCGAATGTATGCACCCTACATGCAAGAACAGTATCAAGAA

181 ATGCTGAAAGTTGCTAATGAACTA**GCACGCCTTCAAGTGTCGTA**TGATGAATTTCTCTGC
iGRQRT4-F

241 ATGAAAGCCTTG**ATGCTCCTCTGTACAATTCCTAAGG**ATGGTTTGAAGAGCCAGGCACTG
iGRQRT4-R

301 TTTGAGGAGATCCGTATGACCTACATCAAAGACTCGG 337

Figure 3.1: Partial cDNA sequence for the newt intracellular glucocorticoid receptor. The nucleotide numbering is arbitrary. The primers used in the final real-time PCR analysis are in bold, with arrows depicting primer directionality.

Relative quantification of gene expression by real-time PCR

The nORL and iGR messenger RNA (mRNA) expression in response to the experimental treatments was quantified using the Applied Biosystems 7700 Real-Time Quantitative PCR system (Perkin-Elmer Applied Biosystems, Foster City, CA). We used the Applied Biosystems SYBR Green PCR Master Mix (Perkin-Elmer) for our real-time PCR reactions. The incorporation of SYBR Green-1 dye into a real-time PCR allows for the detection of any double-stranded DNA generated during the reaction by an increase in fluorescence. The PCR product is quantified at each cycle of the reaction as it accumulates in the exponential phase, before the reaction components become limiting. SYBR green fluorescent signal is collected at each cycle of the reaction and normalized to an internal reference dye (ROX) that does not change during the PCR reaction. The peak-normalized fluorescent values are plotted versus the cycle number. When the reaction is complete, a cycle threshold (C_T) value is assigned to each sample. The C_T is a unitless value defined as the fractional cycle number at which the sample fluorescence signal passes a fixed threshold above baseline. The threshold (10

standard deviations above the mean baseline fluorescence) is automatically set by the software at the completion of the run. The higher the starting concentration of cDNA, the lower the C_T.

The real-time PCR reactions were set up according to the SYBR Green Master Mix protocol. Each 20 µl reaction contained ~1.0 -10 ng of spinal cord cDNA. Pre-determined optimal primer concentrations used in the reactions were: 300 nM for ORLQRT2F and ORLQRT2R, 300nM for iGRQRT4F and 900nM for iGRQRT4R, and 300nM for both actin primers. ABI PRISM optical tubes and caps were used for reaction assembly. Reactions were analyzed on the ABI PRISM 7700 Sequence Detection System. Thermal cycling parameters were 95°C for 10 min; 40 cycles of 95°C for 15 s, 60°C for 1 min; and hold at 4°C. Every sample reaction was run in triplicate, and the averaged result was used for further analysis.

Real time PCR: standard curve construction and sample analysis

For the relative quantitation of gene expression, standard curves are constructed from samples with known amounts of starting cDNA for each gene of interest and the endogenous control. The standards (40, 4.0, 0.4, 0.04 ng/µl) were prepared by serially diluting a stock of brain cDNA, and then drawing from the same dilutions for all subsequent reactions. Each set of standards was run in duplicate reactions on every reaction plate analyzed, and set up according to the reaction specifications described above. Standard curves are shown in Figure 3.2. Each point on the curve is the average of 8 runs on the 7700.

The relative quantity of the gene of interest for the unknown samples was generated from the standard curves by non-linear regression (Figures 3.2 and 3.3), and normalized to the endogenous control (β-actin) from the same sample (ABI PRISM 7700 Sequence Detection System User Bulletin #2, pg 8). Normalization to an endogenous control compensates for differences in the amount of sample

cDNA added to each reaction, and is based on the underlying assumption that the experimental treatment did not affect the expression of the control gene. The normalized value is unitless. Differences in nORL and iGR gene expression due to treatment were tested by ANOVA (Systat 8.0, Evanston, IL).

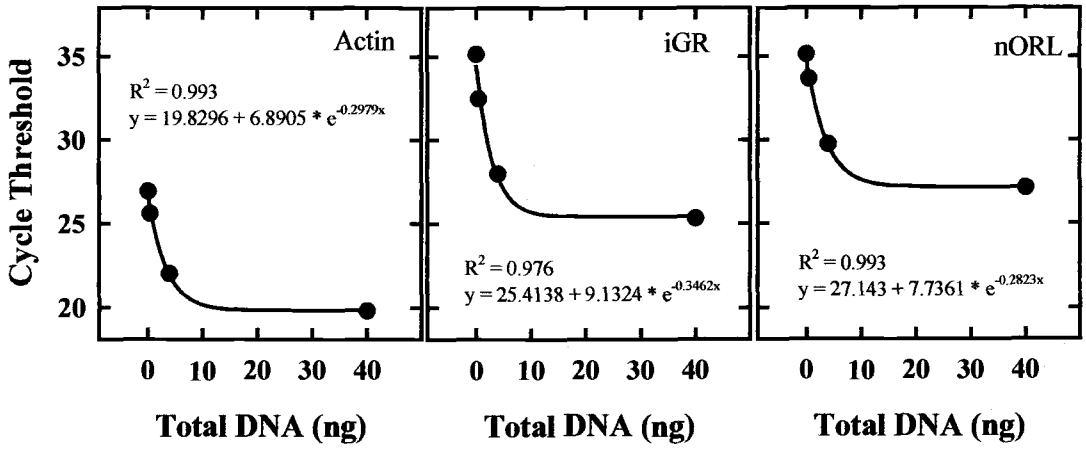

Figure 3.2: Standard curves generated for the quantification of actin, nORL, or iGR gene expression using real-time PCR. Each point is the average of eight observations. Standard errors are smaller than symbol size. The selected standard curve form (3-parameter exponential decay) was chosen based on the best-fit (high R^2 and visual observation) using non-linear regression in Sigma Plot 2000.

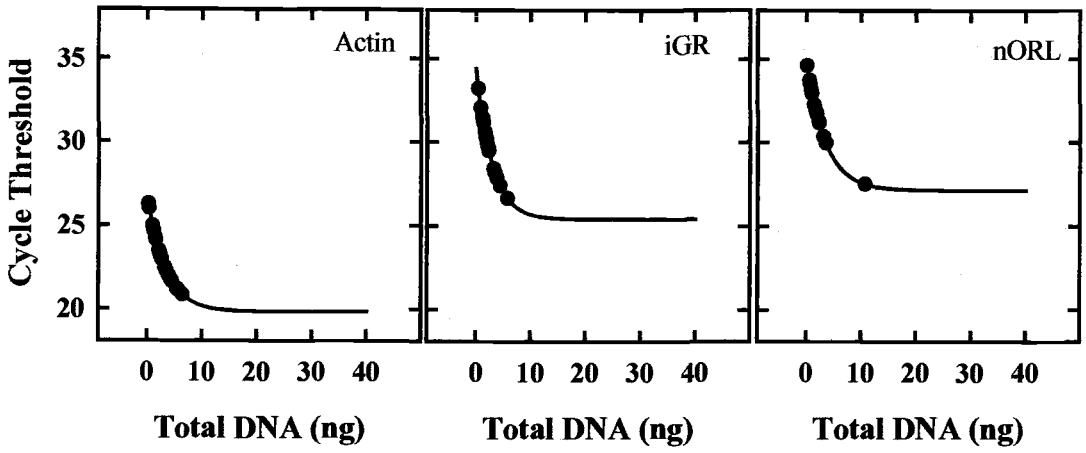

Figure 3.3: Standard curves are shown with the points for the experimental samples superimposed. Samples analyzed for expression generated C_t values that placed them within the range of DNA concentrations used to create the standard curves.

RESULTS

Study A) tissue distribution of the nORL receptor

The expression of the nORL receptor in various tissues was determined by reverse-transcriptase (RT) PCR on newt brain, spinal cord, spleen, small intestine, heart, lung, liver, sperm duct, bladder, and kidney. The cDNA generated from the tissues was tested in PCR reactions using nORL-specific primers. Appropriately sized amplicons (303 bp) were produced in reactions containing cDNA from brain, spinal cord, and lung (Figure 3.4 A). The cDNA from each tissue was also tested in PCR reactions with β-actin primers to ensure the template was of sufficient quality and quantity to amplify. The β-actin primers successfully amplified the cDNA from each tissue, as demonstrated by the appropriately sized amplicon (150 bp) from each reaction (Figure 3.4 B).

Study B) nORL receptor and iGR mRNA expression in the newt spinal cord in response to chronic exposure to corticosterone

Plasma from newts randomly assigned to one of three treatment groups (corticosterone, sham-operated, no-treatment control) was collected following 10 days of treatment. Plasma corticosterone (CORT) levels were analyzed by radioimmunoassay (RIA) to determine if the delivery of hormone was successful in the animals receiving corticosterone-filled capsules, and to determine if the circulating concentrations of CORT in the control animals were within the normal physiological range for non-stressed newts (~0.4-6 ng/ml). The plasma concentration of CORT in the hormone-treated animals was found to be significantly elevated compared to the sham-operated and no-treatment control groups, and was at the upper limit of circulating CORT concentrations found in stressed newts (Figure 3.5). There was no difference between the plasma CORT

levels in the control groups, indicating that the elevation observed in the hormone-treated animals was due to the CORT-implants and was not a by-product of the surgery. The plasma CORT levels in the controls fell within the normal physiological range of non-stressed newts.

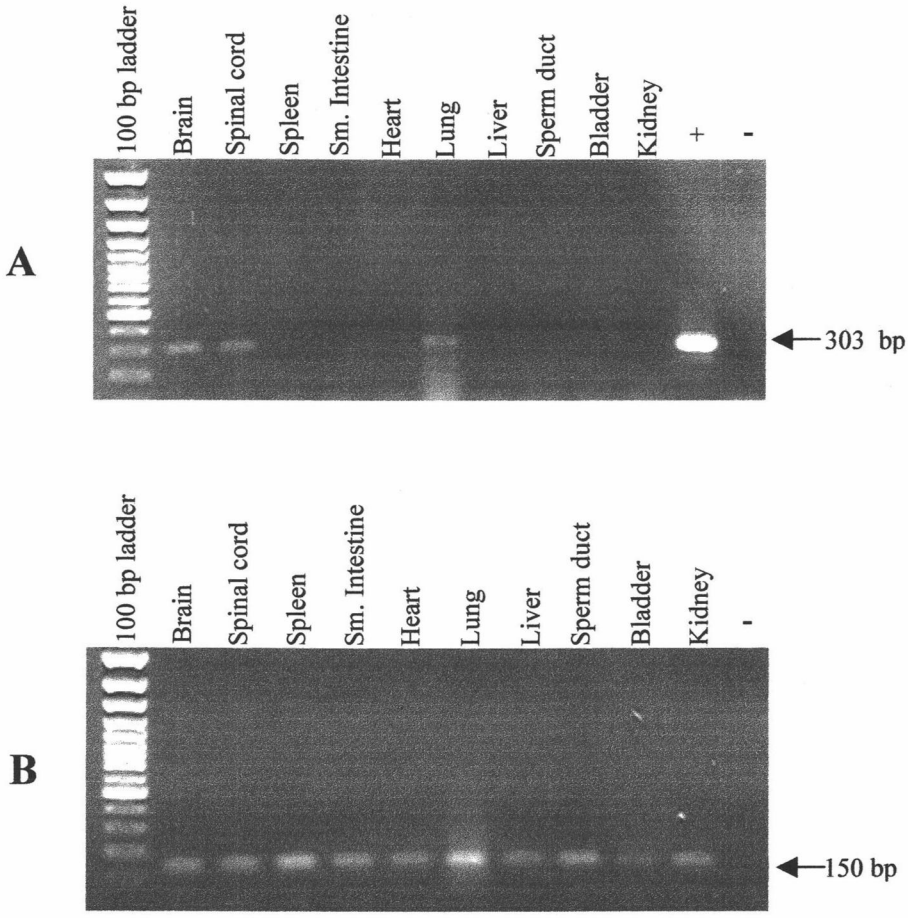

Figure 3.4: Distribution of the nORL receptor as determined by reverse-transcriptase PCR. A) The nORL-specific amplicon (303 bp) was detected in the brain, spinal cord, and lung. B) β-actin primers successfully amplified the appropriately sized fragment (150 bp) from cDNA for all tissues tested, indicating the quality of the template was adequate. +, positive control; -, no template control.

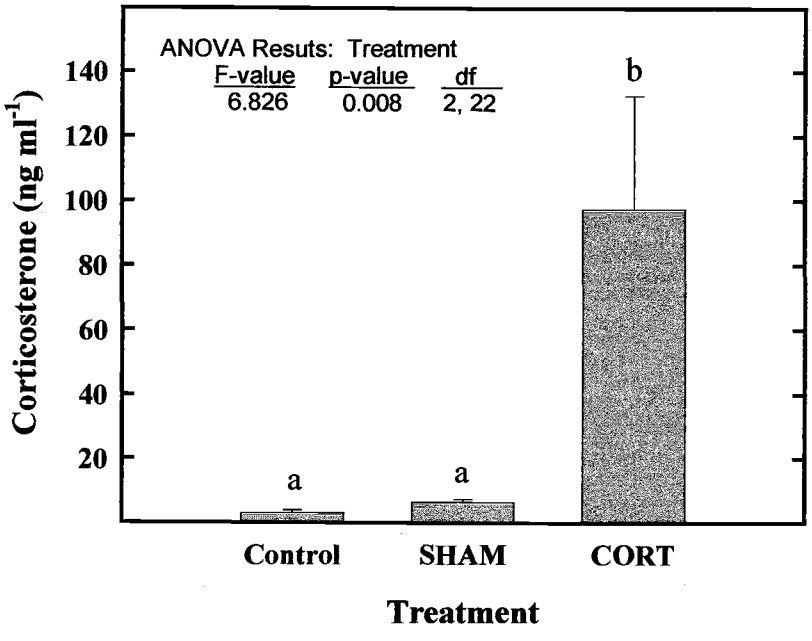

Figure 3.5: Plasma corticosterone (CORT) levels in newts following 10 days of treatment. Each bar represents the mean (n = 6-8) plasma CORT value, and error bars represent one standard error. Animals that received corticosterone implants (CORT) had significantly higher plasma CORT levels than animals in the no-treatment (Control) or sham-operated (SHAM) groups. There was no significant difference between the control groups.

The expression of the nORL and intracellular glucocorticoid receptor messenger RNA (mRNA) in the spinal cord following chronic exposure (10 days) to CORT was examined using real-time quantitative PCR. Standard curves were constructed for each gene of interest, and the relative quantity of each gene in the experimental samples was calculated by interpolation from the standard curves (Figure 3.2). The quantity of gene expression for both receptors was normalized to β-actin

expression, and the normalized values were averaged and compared between treatment groups for differences in expression. There was no significant difference in mRNA expression due to treatment for either the nORL or iGR genes (Figure 3.6).

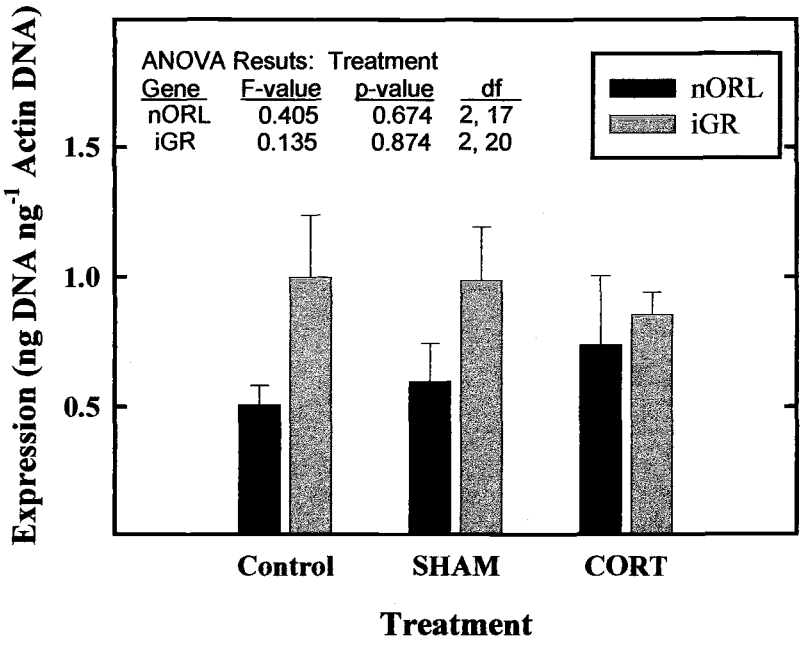

Figure 3.6: The mean (n = 6-8) value for gene expression (nORL or iGR) normalized to β-actin (ng DNA ng^{-1} Actin DNA) is shown for each treatment group. There is no significant difference in expression for either the nORL or iGR genes between groups. Error bars represent one standard error.

DISCUSSION

The results of the reverse-transcriptase (RT) PCR distribution study indicate the nORL receptor is expressed in brain, spinal cord and lung (Figure 3.4). The detection of nORL receptor mRNA in brain and spinal cord was expected, since previous studies have documented high ORL1 receptor densities in these regions (see Mollereau et al. 2000). However, the peripheral distribution of the nORL receptor is unique when compared to the distribution of the mammalian opioid receptor types. nORL receptor mRNA expression was not detected in a number of peripheral tissues shown to express the ORL1 receptor in mammals, including intestine, liver, vas deferens (sperm ducts), and spleen in rat (Wang et al. 1994), and kidney in pig (Osinski et al. 1999). High affinity [^{3}H]nociceptin binding sites on rat heart (Dumont & Lemaire, 1998), and the ability of oFQ to inhibit contractions of rat bladder (Giuliani et al. 1998), also suggest ORL1 receptor expression in these mammalian organs, although none was detected by RT-PCR in the newt. In the present study, the lung was the one peripheral tissue found to contain nORL receptor mRNA. The ORL1 receptor may be expressed in the lungs of mammals as well, since the oFQ peptide can inhibit contractions of guinea pig bronchus when applied to the isolated organ (Rizzi et al. 1999). Based on the limited peripheral distribution of the nORL receptor, it appears its primary role in this species is in central nervous system (CNS) processes. Future studies on the specific distribution of the nORL receptor in brain and spinal cord will provide useful information on inter-species differences and suggest potential roles for the nORL receptor in CNS-mediated behaviors.

The experiments designed to quantify intracellular glucocorticoid receptor (iGR) mRNA in response to chronic exposure to corticosterone (CORT) provided surprising results. We predicted that high circulating plasma CORT levels would result in a downregulation of iGR mRNA due to negative feedback. There is an extremely broad array of experimental evidence supporting the autoregulation of

iGR expression by glucocorticoids, especially the downregulation of protein and mRNA after sustained exposure (see Oakley & Cidlowski, 1993). Instead, we found that chronic exposure to CORT did not influence the expression of iGR mRNA at the level of the spinal cord in the newt (Figure 3.6).

Although the downregulation of iGR expression following exposure to high levels of glucocorticoids is well documented, there do appear to be exceptions to the rule. For example, iGR mRNA is positively regulated following exposure to glucocorticoids in certain cell lines, such as the human CEM lymphoid cell line (Eisen et al. 1988; Antakly et al. 1989), whereas the expression of iGR mRNA is unchanged in the rat hippocampus following CORT administration or chronic stress (Chao et al. 1989; Paskitti et al. 2000). These reported differences in iGR mRNA expression could be due to regulatory mechanisms that are tissue-specific. In a study with similar results to our own, high doses of CORT for 21 days had no affect on iGR mRNA expression in the rat spinal cord, but did result in a decrease in iGR mRNA levels in the hippocampus, although iGR maximum binding capacity (Bmax) at both sites was reduced (Patacchioli et al. 1998). Furthermore, different kinds of stressors appear to have different regulatory effects on iGR mRNA expression. For example, rats exposed to repeated immobilization stress have reduced iGR mRNA levels in the hypothalamic paraventricular nucleus (PVN) and the hippocampus, but iGR mRNA levels in these regions are unchanged after starvation stress, although both treatment groups have high plasma CORT levels (Makino et al. 1995, 2001). In the present study, high circulating levels of CORT did not significantly alter iGR mRNA expression in the newt spinal cord. Whether this lack of affect is due to tissue-specific receptor regulation, method of CORT delivery, or time-course employed requires further investigation.

The regulation of the ORL1 receptor following exposure to high levels of CORT has not been investigated prior to the present study. Previous studies have shown that a link exists between the oFQ system and the stress response in mammals. ORL1 receptor density is known to be high in the mammalian paraventricular

nucleus (PVN) of the hypothalamus, an important source of corticotropin releasing hormone (CRH). The activation of the hypothalamic-pituitary-adrenal (HPA) axis occurs in response to a real or perceived threat, and ultimately results in an increase in circulating levels of glucocorticoids and catecholamines. The activation of ORL1 receptors localized to the PVN would be expected to have an inhibitory effect on the release of neurotransmitters from these cell bodies, since the receptors couple to G_i/G_o mediated transduction systems. Therefore, the oFQ system is potentially a negative modulator of the HPA axis. In support of this role, mice lacking a functional oFQ gene have high basal and post-stress plasma CORT levels, indicating a chronic activation of the HPA axis (Koster et al. 1999). Also, oFQ has anxiolytic properties in rats and mice, and may act to reverse stress-induced behaviors in these animals (Jenck et al. 1997). High levels of circulating glucocorticoids also negatively modulate the activity of the HPA axis by inhibiting the release of CRH and adrenocorticotropic hormone (ACTH) from the hypothalamus and the pituitary. If oFQ and CORT have overlapping functions in the modulation of the stress response, then their respective receptors may be regulated in a parallel manner when an animal is in a chronically stressed state. Therefore, we expected the nORL receptor mRNA expression to mirror the expression of the iGR mRNA following chronic exposure to CORT.

Our results demonstrate that like the iGR, the expression of the nORL receptor mRNA in the spinal cord did not change following chronic exposure to CORT. Opioid receptor regulation, as is the case with the regulation of the iGR, is not a completely understood process. There are many examples of opioid agonists causing receptor downregulation and/or desensitization, but this phenomena is not always accompanied by the expected corresponding decrease in receptor mRNA (see Law et al. 2000). These sorts of results could indicate that the nORL receptor regulation is primarily occurring at the level of the protein, perhaps by a decreased half-life or a reduction in the translatability of the mRNA.

In conclusion, the nORL receptor is expressed in the CNS and the lungs. Our results suggest that chronic stress, as defined by high plasma CORT concentrations over a 10 day period, does not significantly alter the expression of nORL receptor or iGR mRNA at the level of the spinal cord in an amphibian. These results do not preclude the possibility that receptor regulation is occurring post-transcriptionally or post-translationally. These results also have not addressed the regulation of these receptors supraspinally under the same circumstances. Therefore, future studies should focus on mRNA expression in the brain following chronic stress, and on correlating receptor protein levels to mRNA expression.

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CHAPTER 4

SUMMARY

This thesis addressed three primary goals: 1) cloning a complete cDNA sequence for an ORL1-like opioid receptor from an amphibian (*Taricha granulosa*), 2) determining the tissue distribution of the receptor, and 3) examining the regulation of the newt ORL1-like (nORL) receptor following chronic exposure to the glucocorticoid corticosterone. These goals were designed as initial steps towards testing the hypothesis, presented in Chapter 1, that a [^{3}H]CORT binding site identified in roughskin newt neuronal membranes could be located on an opioid-like receptor.

The deduced amino acid sequence for the nORL cDNA is 368 amino acids long, and contains seven hydrophobic regions that resemble the seven membrane-spanning domains characteristic of G protein coupled receptors. The nORL deduced amino acid sequence shares the highest identity (57%) with the human opioid receptor-like (ORL1) receptor, and a fairly equal degree of identity with the human κ, μ, and δ opioid receptors (48-51%). Although the nORL receptor has the highest identity to the ORL1 receptor type, upon closer examination it appears to be a composite of the ORL1 and κ receptor types, alternating between ORL1- and κ- receptor specific residues throughout the sequence. The nORL receptor also differs from the mammalian ORL1 receptor at three specific residues, where it has the conserved classical opioid receptor residues, that may enable it to recognize both ORL1- and κ- receptor selective ligands with high affinity (Meng et al. 1996).

Based on the sequence analysis of the nORL receptor, we predict that the pharmacology of this receptor will differ from its mammalian counterpart. Specifically, we expect the nORL receptor to have affinity for the ORL1 receptor's

endogenous ligand, orphanin FQ (oFQ), as well as prodynorphin peptides and some kappa-selective and non-selective opiates. This predicted pharmacology resembles that of the [^{3}H]CORT binding site discussed in Chapter 1. Therefore, we hypothesize that the [^{3}H]CORT binding site may be located on the nORL receptor. This hypothesis will be tested by expressing the nORL receptor in a mammalian tissue culture system, and performing radioligand binding studies on cells expressing the receptor.

The nORL receptor is expressed in the brain, spinal cord, and lungs of the newt. The distribution of the nORL receptor was determined by reverse-transcribing RNA from a variety of tissues into cDNA, and amplifying the cDNA with nORL receptor-specific primers. The peripheral distribution of the nORL receptor does not match the reported peripheral distribution for any of the mammalian opioid receptor types, including its closest homologue, the ORL1 receptor. Therefore, the role of the nORL receptor in the newt periphery could differ from the role of homologous receptors in mammals. The localization of nORL receptor mRNA to the brain and spinal cord supports a role for this receptor in the modulation of the central nervous system (CNS) circuitry, as proposed for opioid receptors in mammals. The expression of the nORL receptor in the spinal cord also supports the hypothesis that CORT may act through an opioid-like receptor in the newt to rapidly affect behavior; CORT is able to inhibit clasping in spinally transected newts, indicating CORT can act at the level of the spinal cord to control this behavior.

The logic behind investigating the regulation of the nORL receptor following chronic exposure to CORT was two-fold: 1) As discussed in Chapter 3, a potential role for the oFQ/ORL1 system is in the modulation of the stress response, and since CORT is a classic stress hormone, we predicted that nORL receptor regulation would be affected following chronic exposure to CORT; and 2) As discussed in Chapter 1, it has been hypothesized that a [^{3}H]CORT binding site localized to newt neuronal membranes may be on an opioid-like receptor (Evans et al. 2000);

therefore, we predicted that if CORT was binding directly to the nORL receptor, chronic exposure to CORT would result in down-regulation of the receptor, as shown for characterized glucocorticoid receptors previously.

To test these hypotheses, we exposed newts to CORT for 10 days by surgically implanting Silastic capsules packed with CORT powder. Control animals received empty capsules, or no treatment. The spinal cords of the animals were collected at the completion of the hormone treatment, and the RNA was extracted and reverse-transcribed into cDNA. The cDNA was used in real-time PCR reactions for the indirect relative quantitation of the nORL receptor mRNA. The same cDNA samples were also used for the relative quantitation of mRNA for the intracellular glucocorticoid receptor (iGR). The iGR mRNA was quantitated because the iGR is a known receptor for CORT. Therefore, our intent was to compare the regulation of the nORL receptor to the regulation of the iGR receptor.

The results of the real-time PCR experiment indicate that neither the nORL receptor mRNA nor the iGR mRNA levels differ from the control levels following chronic exposure to CORT. Since the iGR is a known receptor for CORT, these results indicate that chronic exposure to agonist does not cause a decrease in the mRNA expression of the iGR in newt spinal cord. Furthermore, chronic exposure to CORT also has no effect on the mRNA expression of the nORL receptor in the spinal cord. These studies did not address the regulation of the receptors at the level of protein, so it is possible that receptor regulation could be occurring through a pre- or post-translational mechanism. This possibility awaits further investigation. This study does demonstrate that the nORL receptor and the iGR are regulated at the transcriptional level, as determined by mRNA levels, in a parallel manner following chronic exposure to CORT at the level of the newt spinal cord.

In conclusion, this is the first time a full-length cDNA sequence for an opioid-like receptor has been obtained from an amphibian. These studies have provided new information on the sequence of an opioid-like receptor, its distribution, and its regulation following chronic exposure to a stress hormone in an amphibian.

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