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Delta and mu opioid receptors from the brain of a urodele amphibian, the rough-skinned newt *Taricha granulosa*: Cloning, heterologous expression, and pharmacological characterization

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Abstract

Two full-length cDNAs, encoding delta (δ) and mu (μ) opioid receptors, were cloned from the brain of the rough-skinned newt *Taricha granulosa*, complementing previous work from our laboratory describing the cloning of newt brain kappa (κ) and ORL1 opioid receptors. The newt δ receptor shares 82% amino acid sequence identity with a frog δ receptor and lower (68–70%) identity with orthologous receptors cloned from mammals and zebrafish. The newt μ receptor shares 79% sequence identity with a frog μ receptor, 72% identity with mammalian μ receptors, and 66–69% identity with μ receptors cloned from teleost fishes. Membranes isolated from COS-7 cells transiently expressing the newt δ receptor possessed a single, high-affinity ($K_d = 2.4$ nM) binding site for the nonselective opioid antagonist [^{3}H]naloxone. In competition binding assays, the newt δ receptor displayed highest affinity for Met-enkephalin, relatively low affinity for Leu-enkephalin, β-endorphin, and [D-penicillamine, D-penicillamine] enkephalin (DPDPE) (a δ-selective agonist in mammals), and very low affinity for μ-, κ-, or ORL1-selective agonists. COS-7 cells expressing the newt μ receptor also possessed a high-affinity ($K_d = 0.44$ nM) naloxone-binding site that showed highest affinity for β-endorphin, moderate-to-low affinity for Met-enkephalin and Leu-enkephalin and DAMGO (a μ-selective agonist in mammals), and very low affinity for DPDPE and κ- or ORL1-selective agonists. COS-7 cells expressing either receptor type (δ or μ) showed very high affinity ($K_d = 0.1$–0.3 nM) for the nonselective opioid antagonist diprenorphine. *Taricha granulosa* expresses the same four subtypes (δ, μ, κ, and ORL1) of opioid receptors found in other vertebrate classes, but ligand selectivity appears less stringent in the newt than has been documented in mammals.

Keywords: Delta; Mu; Opioid receptor; G protein; Brain; Amphibian; Salamander; Newt

1. Introduction

Among the first neuropeptides to be identified in the central nervous system were the endogenous opioids, including the enkephalins (Hughes et al., 1975), dynorphins (Goldstein et al., 1979), and β-endorphin (Bradbury et al., 1975). These endogenous peptides and their synthetic analogs, as well as naturally occurring alkaloid derivatives of opium, and synthetic benzomorphans (Goldstein and Naidu, 1989) and arylacetamides (Lahti et al., 1982, 1985; Piercey et al., 1982), bind selectively to specific opioid receptors that are widely distributed in the brain and spinal cord as well as in the peripheral nervous tissues (recent reviews by Janecka et al., 2004; Waldhoer et al., 2004). Activation of opioid receptors by endogenous or exogenous ligands can lead to modulation of multiple and diverse physiological functions and/or behaviors, including analgesia/nociception, hormone secretion, neurotransmitter release, gastrointestinal motility, respiratory control, thermoregulation, food consumption, effects on or related to anxiety, and feelings of euphoria—which, in turn, can lead to opioid tolerance and dependence (Akil et al., 1984; Pasternak, 1988; Waldhoer et al., 2004).

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Four different opioid receptor subtypes are now widely recognized: the δ-opioid receptor (DOR, named for its discovery in the mouse vas deferens), the μ-opioid receptor (MOR, named for the prototypic ligand morphine—itself named after Morpheus, the Greek god of dreams), the κ-opioid receptor (KOR, named for the prototypic ligand ketocyclazocine), and ORL1 (named because it was originally judged 'opioid receptor like')—the nociceptin/orphanin FQ receptor. All four subtypes of opioid receptors belong to the superfamily of G-protein-coupled receptors that possess seven transmembrane domains (Akil et al., 1996; Raynor et al., 1996), and all have been shown to be negatively coupled to adenylate cyclase by way of pertussis toxin-sensitive GTP-binding regulatory proteins (Reisine and Bell, 1993). Opioid ligand–receptor interaction results in the activation of an inwardly rectifying K^+ conductance and in the inhibition of voltage-gated Ca^{2+} channels (Ikeda et al., 2002; New and Wong, 2002; Williams et al., 2001).

Comparative studies suggest that the opioid receptor gene family arose early in vertebrate evolution and has been highly conserved over time (Barrallo et al., 2000; Darlison et al., 1997). Full-length opioid receptor cDNA sequences cloned from a variety of mammalian species (human, monkey, mouse, rat, cow, pig, guinea pig) have been deposited in the GenBank database, and partial sequences of genes belonging to the opioid receptor family have been identified in birds, amphibians, and fish—including the jawless Pacific hagfish (*Eptatretus stoutii*), which is considered as an ancient lineage occupying a key position in vertebrate evolution (Li et al., 1996a,b). Despite the fact that many of the earliest studies describing the isolation, solubilization, purification, and partial characterization of opioid receptors were conducted in amphibians, specifically, using membranes prepared from the brains of toads (*Bufo marinus*) (Ruegg et al., 1980, 1981; Simon et al., 1982) or frogs (*Rana pipiens*) (Benyhe et al., 1994; Borsodi et al., 1986; Mollereau et al., 1988; Simon et al., 1984, 1985, 1987, 1990), the vast majority of reports describing the molecular cloning and pharmacological characterization of full-length opioid receptors come from studies of mammals. Among nonmammalian vertebrates, a full-length cDNA sequence for a putative zebrafish DOR has been cloned (Barrallo et al., 1998) and characterized with respect to ligand-binding properties (Rodriguez et al., 2000), and full-length cDNA sequences for MORs have been reported for the white sucker (Darlison et al., 1997) and zebrafish (Barrallo et al., 2000). μ, κ, and δ-opioid-binding sites have been described in whole brain homogenates prepared from the brain of the grass frog (*R. pipiens*) (Newman et al., 2002), and full-length DOR and MOR sequences from *R. pipiens* have been deposited in GenBank (Accession Nos. AF530572 and AF530571, respectively), but ligand-binding properties of cloned sequences from amphibians have so far not been described or otherwise matched with pharmacological properties observed for binding sites present in the brain.

In recent reports, our laboratory has described the cloning, heterologous expression, and pharmacological characterization of a κ-opioid receptor (Bradford et al., 2005) and an ORL1 receptor (Walthers et al., 2005) present in the brain of the rough-skinned newt, *Taricha granulosa*. In this article, we present the results of similar studies undertaken to identify and characterize the newt brain DOR and MOR. Demonstration that newts possess these two opioid receptor subtypes is consistent with previous work in our laboratory, demonstrating (1) that morphine (presumably acting through a μ-type binding site) attenuates corticotropin-releasing factor-induced locomotor activity in newts (Lowry et al., 1990) and (2) that the newt brain expresses a mRNA for proenkephalin, the precursor for the presumed endogenous ligand(s) for the DOR (Walthers and Moore, 2005). *Taricha granulosa* is the first nonmammalian vertebrate for which cDNAs representing each of the four opioid receptor subtypes have been cloned, expressed in a heterologous system, and characterized pharmacologically.

2. Materials and methods

2.1. Animals

Newts were collected from local (Benton County, OR, USA) freshwater ponds. All animals were housed, fed, and handled in accordance with federal and institutional guidelines.

2.2. Degenerate polymerase chain reaction

Full-length cDNA sequences available from the GenBank database for DOR and MOR cloned from various species were aligned using ClustalW, and degenerate oligonucleotide primers were designed using Primer3 software (Rozen and Skaletsky, 2000) based on highly conserved regions in transmembrane domains I and VI (TMI and TMVI). The sequences of the sense and the antisense primers (Integrated DNA Technologies, Coralville, IA, USA) were 5′-ATCGCCATCACCGCGCTCTAC-3′ and 5′-CACM ACCACCAGCACCATGC-3′ (for δ), and 5′-GGAAACTTCCTGGTCA TGTATGTGAT-3′ and 5′-ATGTGRATGGGRGTCCAGCA-3′ (for μ). Total RNA was prepared from the pooled brains of six adult male newts according to the protocol supplied with the ToTALLY RNA kit (Ambion, Austin, TX, USA). This procedure was performed several times, on different dates, to obtain multiple RNA pools prepared independently. cDNA was obtained by reverse transcriptase-polymerase chain reaction (RT-PCR) using a cDNA first strand synthesis kit (Fermentas, Hanover, MD, USA). This procedure was performed several times, on different dates, using different RNA templates. For degenerate PCR designed to amplify a partial (TMI–TMVI) newt DOR (nDOR), primers, dNTPs, *Taq* polymerase, and newt brain cDNA template were used in PCR reactions with the following cycling conditions: 94 °C for 3 min; four cycles of 94 °C for 45 s, 50–60 °C (gradient) for 45 s, 72 °C for 1 min; 30 cycles of 94 °C for 30 s, 50–60 °C for 30 s, 72 °C for 1 min; terminal extension at 72 °C for 8 min. For degenerate PCR designed to amplify a partial (TMI–TMVI) newt MOR (nMOR), the following cycling conditions were used: 94 °C for 3 min; four cycles of 94 °C for 45 s, 60 °C for 45 s, 72 °C for 1 min; 30 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min; terminal extension at 72 °C for 7 min. Negative controls included autoclaved water in place of the cDNA template. PCR products were separated on a 1.4% agarose gel, stained with ethidium bromide, and visualized with UV light. PCR products of the appropriate size (~653 bp for δ; ~642 bp for μ) were purified from gels using a GelSpin DNA Purification Kit (MO BIO, Solana Beach, CA, USA), ligated