Cloning, heterologous expression and pharmacological characterization of a kappa opioid receptor from the brain of the rough-skinned newt, *Taricha granulosa*

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Abstract

A full-length cDNA that encodes a kappa (κ) opioid receptor has been isolated from the brain of a urodele amphibian, the rough-skinned newt *Taricha granulosa*. The deduced protein contains 385 amino acids and possesses features commonly attributed to G protein-coupled receptors, such as seven putative transmembrane domains. The newt κ receptor has 75% sequence identity to κ opioid receptors cloned from mammals, and 66% sequence identity to the κ opioid receptor reported for the zebrafish, with the greatest divergence in the extracellular N-terminus, the second and third extracellular loops and the intracellular C-terminus. Membranes isolated from COS-7 cells expressing the newt κ receptor possessed a single, high-affinity ($K_d=1.5$ nM) binding site for the κ -selective agonist U69593. In competition binding assays, the expressed newt κ receptor displayed high affinity for the κ -selective agonists GR89696, dynorphin A(1–13), U69593, U50488 and BRL52537, as well as the κ -selective antagonist nor-binaltorphimine and the non-selective antagonist naloxone. Rank order potencies and affinity constants were similar in competition binding studies that used either whole brain homogenates or membranes isolated from COS-7 cells expressing the newt κ receptor. The expressed receptor displayed essentially no affinity for the δ -selective agonist DPDPE ([d-penicillamine, d-penicillamine]enkephalin), but showed moderate affinity for the μ -selective agonist DAMGO ([d-Ala-MePhe, Gly-ol]enkephalin) and moderately high affinity for nociceptin (orphanin FQ), the endogenous ligand for the opioid receptor-like (ORL)1 receptor. These findings support the conclusions that a gene for the κ opioid receptor is expressed in amphibians and that the pharmacology of the newt κ receptor closely matches mammalian κ opioid receptors. However, the functional dichotomy between the classic opioid receptors (κ , δ , μ) and ORL1 appears less strict in amphibians than in mammals.

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Introduction

Kappa (κ), delta (δ), mu (μ) and ORL1 (opioid receptor-like) represent the four main classes of opioid receptors, which are defined and distinguished by primary structures, anatomical distributions, pharmacological profiles and physiological functions. Opioid receptors belong to the superfamily of G protein-coupled receptors possessing seven transmembrane domains (Janecka *et al.* 2004, Waldhoer *et al.* 2004), and when activated, typically produce an inhibition of adenylyl cyclase activity (Reisine & Bell 1993) and the modulation of calcium and potassium conductances (Darlison *et al.* 1997).

κ opioid receptors (named for the prototypic ligand ketocyclazocine) selectively bind endogenous peptides derived from pro-dynorphin (Chavkin *et al.* 1982), as well as synthetic benzomorphans (Goldstein & Naidu 1989) and arylacetamides such as U50488 (Lahti *et al.* 1982, Piercey *et al.* 1982) and U69593 (Lahti *et al.* 1985). κ receptors participate in a wide range of physiological

functions, including pain perception, feeding and drinking behavior, water balance, gut motility, temperature regulation and cardiovascular control (Bloom 1983). Because activation of κ receptors produces analgesia in the absence of primary reinforcement (Meng *et al.* 1993), the κ receptor has been an attractive target for development of analgesic drugs with low abuse potential. Although early κ agonists evaluated in humans showed a predisposition for eliciting central nervous system disturbances (altered perception, dysphoria, psychotomimesis) (Pfeiffer *et al.* 1986), κ -related drugs developed more recently do not provoke such adverse side effects and have been demonstrated to be neuroprotective and potentially effective in the treatment of stroke and ischemic brain attacks (Tortella *et al.* 1997).

Many early studies describing the isolation, solubilization, purification and partial characterization of κ opioid receptors were conducted using membranes prepared from the brains of toads (*Bufo marinus*) (Ruegg *et al.* 1980, 1981, Cone & Goldstein 1982, Simon *et al.* 1982) or frogs (*Rana pipiens*) (Benyhe *et al.* 1994, Simon *et al.* 1984,

1987, 1990, Stevens *et al.* 1987), because κ receptors were found to be highly abundant in the amphibian central nervous system. Early experiments with frogs and toads demonstrated the presence of immunoreactive dynorphin in the brain (Cone & Goldstein 1982) and reported antinociceptive responses to exogenously administered dynorphin (Stevens *et al.* 1987). In contrast, most studies describing molecular cloning of κ opioid receptors have focused on mammalian species; nucleic acid/amino acid sequence data correlated with binding pharmacology for non-mammalian species are non-existent.

To date, full-length cDNA sequences have been reported for mouse (Yasuda *et al.* 1993), rat (Chen *et al.* 1993, Meng *et al.* 1993, Minami *et al.* 1993, Yakovlev *et al.* 1995), guinea pig (Xie *et al.* 1994) and human (Mansson *et al.* 1994, Simonin *et al.* 1995); a partial sequence for a bovine κ receptor (Li *et al.* 1996) is also available. Partial sequences for κ opioid receptors have been reported for two teleost fishes, white sucker (*Catostomus commersoni*) (Li *et al.* 1996, Darlison *et al.* 1997) and bass (*Morone saxatilis*) (Li *et al.* 1996), and a full-length cDNA sequence for a zebrafish (*Danio rerio*) κ receptor ('ZFOR3') has been deposited in the GenBank database (Accession AF285173) along with the predicted amino acid sequence (GenBank Accession NP_878306). Among avian species, a κ opioid receptor has been identified and partially characterized pharmacologically in a passerine songbird (*Junco hyemalis*) (Deviche *et al.* 1993). Among amphibians, a cDNA sequence for a κ -like opioid receptor from the northern grass frog (*R. pipiens*) has been deposited in the GenBank database (Accession AF530573), but this sequence includes a base insertion that leads to a frameshift in the potential coding region. For this reason the identification or status of any protein(s) encoded by the grass frog cDNA sequence remains unclear.

Among urodele amphibians, no cloning experiments directed at κ opioid receptors have been reported to date, despite compelling evidence for the existence and physiological relevance of κ receptors in these animals. In male rough-skinned newts (*Taricha granulosa*), ethylketocyclazocine and bremazocine (κ -selective receptor agonists) suppress sexual behavior (amplectic clasping of females); the effect of bremazocine is reversed in a dose-dependent fashion by treatment with naloxone (a non-selective opioid antagonist) (Deviche & Moore 1987). Bremazocine suppresses spontaneous (Deviche *et al.* 1989) but not corticotropin-releasing factor-induced (Lowry *et al.* 1990) locomotor activity in male newts, supporting the idea that opioids may be important regulators of amphibian behavior. Finally, some but not all κ -selective agonists are capable of displacing [^3H]corticosterone from the membrane glucocorticoid receptor identified in the *Taricha* brain (Evans *et al.* 2000). This membrane steroid receptor mediates rapid

behavioral responses, such as suppression of amplexic clasping (Orchinik *et al.* 1991, Moore & Orchinik 1994), through a mechanism that is modulated by Mg^{2+} and guanyl nucleotides (Orchinik *et al.* 1992). Together, these observations have prompted speculation that the high-affinity membrane binding site for corticosterone is, in fact, located on a κ -like opioid receptor (Evans *et al.* 2000).

The current study was undertaken in an effort to characterize the κ opioid receptor(s) present in the brain of a urodele amphibian, the rough-skinned newt *T. granulosa*. Here we report the molecular cloning and heterologous expression of a full-length cDNA sequence that encodes the newt brain κ opioid receptor (nKOR). The deduced amino acid sequence of nKOR was compared with the other full-length κ opioid receptors that have been cloned to date. Binding pharmacology was characterized for nKOR transiently expressed in COS-7 cells, and compared with the results of similar binding assays performed using synaptosome preparations isolated from whole newt brains. This is the first time a non-mammalian κ opioid receptor has been cloned, expressed in a heterologous system, and characterized.

Materials and methods

Animals

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

Degenerate PCR

Full-length cDNA sequences available from the GenBank database for various species (human, mouse, rat, pig, guinea pig, zebrafish) were aligned using ClustalW, and degenerate oligonucleotide primers were designed using Primer3 software (Rozen & Skaletsky 2000) based on highly conserved regions in the N-terminus and first intracellular loop. The sequences of the sense and the antisense primers (purchased from The Great American Gene Company) were 5'-GCACAT CTCYCCRGCCATCC-3' and 5'-GGTCCACRCTCA TCATGGTCAA-3' respectively. 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, in order to obtain multiple RNA pools prepared independently. cDNA was obtained by 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, primers, dNTPs, Taq polymerase and newt brain cDNA template were used in PCR reactions with the following cycling conditions: 94 °C × 3 min; 4 cycles of 94 °C × 45 s, 55 °C × 45 s, 72 °C × 1 min 30 s; 34 cycles of 94 °C × 30 s, 55 °C × 30 s, 72 °C × 1 min; terminal extension at 72 °C × 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 (~309 bp) were purified from gels using a GelSpin DNA Purification Kit (MO BIO, Solana Beach, CA, USA), ligated into the pCR4-TOPO vector (Invitrogen), and transformed into TOP10 *Escherichia coli* competent cells. Following overnight incubation, plasmid DNA was isolated from minipreps and purified by alkaline lysis using a Qiaprep kit (Qiagen). Purified DNA was sequenced by the Central Services Laboratory at Oregon State University. Sequences were analyzed with a BLAST-N 2.0 search tool provided by NCBI.

Rapid amplification of cDNA ends (RACE)-PCR

Clones identified by degenerate PCR possessing the highest sequence identity to known κ opioid receptors were used to design gene-specific primers for RACE-PCR. The sense primer (for 3'RACE) was 5'-GGGA ACGTGCTCTGTAAATAGTC-3'; the antisense primer (for 5'RACE) was 5'-CATGACCAAGGAGT TTCCCACCAG-3'. Gene-specific primers were purchased from One Trick Pony (Ransom Hill Bioscience, Ramona, CA, USA). 3'RACE-ready cDNA was prepared from newt brain RNA according to the FirstChoice RLM-RACE kit protocol (Ambion). 3'RACE-ready cDNA was used in conjunction with the nKOR gene-specific sense primer and the Ambion 3' 'Inner' antisense primer in a 3'RACE-PCR reaction according to the kit protocol and recommended cycling conditions. Following agarose gel electrophoresis, a PCR product (approximately 1.4 kb) was gel purified, sub-cloned, and sequenced as described above.

5'RACE-ready cDNA was synthesized from newt brain RNA according to Ambion's RLM-RACE kit protocol. 5'RACE-PCR reactions were carried out using the Ambion kit reagents and protocol, the Ambion 'Outer RACE' sense primer and a 5'RACE gene-specific antisense primer with the following cycling conditions: 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 × 7 min.

Regions of overlapping and identical sequences among the original degenerate PCR product and the 5'RACE and 3'RACE products were joined using SeqMerge software (Accelrys, San Diego, CA, USA) to produce a contiguous sequence representing the

full-length nKOR cDNA. New gene-specific primers were designed at the extreme 5' and 3' ends of the open reading frame, and the complete coding region was subsequently PCR amplified using a high-fidelity Pfx proof-reading polymerase (Invitrogen) and sub-cloned three separate times to obtain a consensus sequence.

Phylogenetic tree construction

Available full-length opioid receptor protein sequences for species other than *T. granulosa* were obtained from GenBank. Amino acid sequences were aligned using ClustalX (v1.81) (Thompson *et al.* 1997). The alignment was analyzed using the bootstrap and protein sequence parsimony components of PHYLIP 3.6 (Felsenstein 1989, 2004) to generate a phylogenetic tree. The tree was rooted in *Rattus norvegicus* somatostatin receptor type 1, representing a family of G protein-coupled receptors closely related to the opioid receptors (Pfeiffer *et al.* 2002).

Transient expression of nKOR in COS-7 cells

The full-length nKOR cDNA was sub-cloned into the mammalian expression vector pcDNA3.1/V5-His-TOPO (Invitrogen) and individual clones were analyzed for correct orientation with respect to the CMV promoter. COS-7 (African green monkey kidney) cells (American Type Culture Collection, Manassas, VA, USA) were cultured in 10 cm tissue culture plates at 37 °C under 5% CO₂ in Dulbecco's Modified Eagle's Medium (Mediatech Cellgro, Herndon, VA, USA) supplemented with 2 mM L-glutamine (Invitrogen) and 10% fetal bovine serum (Hyclone, Logan, UT, USA). Transient expression was achieved by transfecting plates (90–95% confluency) using Lipofectamine 2000 reagent (Invitrogen) diluted with Opti-MEM medium (Invitrogen). For each 10 cm culture plate, 24 µg DNA in 1.5 ml Opti-MEM and 60 µl Lipofectamine 2000 in 1.5 ml Opti-MEM were added to a plate containing 12 ml growth medium. Six hours after transfection, the lipofection mixture was aspirated and replaced with 14 ml of fresh growth medium.

Preparation of COS-7 cell membranes

Forty-eight hours after transfection, cells were washed twice with room temperature PBS and harvested under homogenization buffer (2 ml/plate) using a cell scraper. Suspended cells were homogenized in chilled buffer (25 mM Hepes, 100 µM phenylmethylsulfonyl fluoride (PMSF), 100 µg/ml trypsin inhibitor, 0.7 µg/ml leupeptin, 100 µg/ml bacitracin) using a chilled glass-on-glass Dounce homogenizer. Homogenates were centrifuged

for 10 min at 45 000 *g* at 4 °C. Supernatants were discarded and pellets were resuspended in homogenization buffer (1 ml/plate) and centrifuged a second time. Supernatants were discarded and cell membrane pellets were resuspended in binding buffer (250 µl/plate) containing MgCl₂ (see Binding Assays described below), snap-frozen in liquid N₂, and stored at -80 °C until use. In the course of this study, multiple transfections were performed; however, tissues from different transfections were not pooled, and in a given binding assay all tissues used were obtained from a single transfection. The concentration of total protein in membrane preparations was determined using a colorimetric protein assay (Pierce, Rockford, IL, USA).

Preparation of neuronal P2 membranes from whole newt brains

Neuronal membranes (P2 membrane pellets) were prepared according to the method of Whittaker (1969), as modified by Orchinik *et al.* (1991). Brains were removed from anesthetized adult male newts and placed in 0.3 ml of chilled homogenization buffer (0.32 M sucrose, 5 mM HEPES, pH 7.45, 100 µM PMSF) per mg wet tissue weight. Brains were homogenized in a Teflon-on-glass Dounce homogenizer. The homogenate was centrifuged at 1000 *g* for 10 min at 4 °C, after which the supernatant was transferred to a clean centrifuge tube. The pellet (P1) was resuspended in the original volume of homogenization buffer and centrifuged again, as above. The new supernatant was added to the first supernatant and the pellet discarded. Pooled supernatants were centrifuged at 30 000 *g* for 40 min at 4 °C. The pellet (P2) was snap-frozen in liquid N₂, thawed on ice, and resuspended in 0.18 ml/mg (original tissue weight) of 25 mM HEPES, 10 mM EDTA, pH 7.45, 100 µM PMSF. The suspension of P2 was incubated at 4 °C for 2 h, to allow for dissociation of endogenous ligand(s), and centrifuged at 40 000 *g* for 15 min at 4 °C. Supernatant was discarded and the final pellet was snap-frozen in liquid N₂ and stored at -80 °C.

Binding assays

Saturation and competition binding assays were performed in buffer consisting of 25 mM Hepes (pH 7.45) containing 10 mM MgCl₂, 100 µM PMSF, trypsin inhibitor (100 µg/ml), leupeptin (0.7 µg/ml) and bacitracin (100 µg/ml). Frozen membrane pellets (COS-7 or P2) were thawed on ice and diluted with binding buffer to achieve a final protein concentration of 50 µg per binding reaction. For saturation experiments, various concentrations of [³H]U69593 (41.4 Ci/mmol; Perkin Elmer Life Sciences, Boston, MA, USA) were used in the presence of 10 µM nor-binaltorphimine (nor-BNI) (TOCRIS, Ellisville, MO, USA). Competition

binding experiments were performed using 1–2 nM [³H]U69593 and various doses (10⁻¹² to 10⁻⁴ M) of non-radioactive competitors. GR89696, U50488, BRL52537 and [D-Ala-MePhe, Gly-ol]enkephalin (DAMGO) were purchased from TOCRIS; dynorphin A(1–13) amide was from Phoenix Pharmaceuticals (Belmont, CA, USA); U69593 was from Sigma-Aldrich; nociceptin and [D-penicillamine, D-penicillamine] enkephalin (DPDPE) were from Multiple Peptide Systems (San Diego, CA, USA). Non-specific binding was defined as radioactivity remaining bound in the presence of 10 µM non-radioactive U69593. Triplicate samples were run at each dose for both total and non-specific binding. Assays were conducted at 4 °C for 4 h with gentle mixing (80 r.p.m. on an orbital shaker). At the end of the incubation period, bound and free ligand were separated by rapid filtration over GF/C filters (pre-soaked with 0.25% polyethyleneimine for 30 min in inhibition experiments with peptide ligands) under vacuum using a Brandel (Gaithersburg, MD, USA) cell harvester. Filters were washed twice with 4 ml of chilled (4 °C) Hepes/MgCl₂ (pH 7.45) buffer. Radioactivity in the filters was determined by liquid-scintillation counting on a Beckman LS 6500 scintillation counter. Binding data were analyzed using GraphPad Prism software (GraphPad Software, San Diego, CA, USA).

Results

cDNA and deduced amino acid sequence analyses

The degenerate PCR procedure yielded an amplified product of 269 bp, representing a cDNA sequence encoding a peptide of 81 amino acids. BLAST analysis of the deduced amino acid sequence predicted a hypothetical protein characterized by putative conserved domains consistent with membership in the family of proteins containing seven transmembrane domains (rhodopsin family), and showed highest sequence identity to mammalian κ opioid receptors. RACE-PCR yielded additional nucleotide sequences overlapping the original 'core' sequence at each end, and allowed construction of a single, contiguous, nucleotide sequence.

The contiguous cDNA amplified from degenerate and RACE-PCR performed using newt brain cDNA template is shown in Fig. 1. This sequence contains an open reading frame spanning nucleotides 1–1158. The sequence of the open reading frame was independently verified at least three times, using different preparations of newt brain cDNA prepared independently of one another. No products were amplified in negative controls that included autoclaved water in place of the cDNA template. Immediately upstream of the putative start codon shown in Fig. 1 is the sequence GTTATGG, a variation on the Kozak consensus sequence (for efficient initiation of transcription) (G/A)NNATGG


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-217   aaaagaatccagcagcactcctcgcccccagctctc -181
aggagggatcagcgcgggaggagagctcgaggagctgggtggccagaagccctcgctg -121
tacctgaggggggtgtccccggataggagcacgcctcgcaaccttccagaccaacagtc -61
ttgaccaaagggtcggtgtgtctcacacgcacaggaagagggcaggcggtcaggtt -1

M E S P V Q I F R E K E Q A L E A G R L      20
atggagtcaccctgcatgtttcagagagaaagaacaggcgctagaggcgggcgctg      60
      ●
E P T C C V P T N S S I W P L G W H D Y      40
gagccacactgtgctgctcccaaccaactccagcatctggcctctgggtggcagcactac      120
      ●
Y G L N G S E E A W E L G H H L A N H T      60
tatgggtcctaatggcagcgaagaggcctgggagctaggccatcacctagccaaccatacc      180

      TMI
N I S P T I P I I I T A V Y S M V F V V      80
aacatatccccaccatccccatcatcatcaccgcagtctactccatggtctttgtggtc      240

G L V G N S L V M F V I I R Y T K M K T      100
ggcctgggtgggaaactccttggtcatgttcgtcatcatcagggtatacaaaagatgaagacg      300
TMI I
A T N I Y I F N L A M A D A L V T T T M      120
gcaacaaacatctatatatttttaattggcaatggcggacgcttggttacacgacaatg      360
      ◆
P F Q S T E Y L M N S W P F G N V L C K      140
Ccatttcaaaagcaccgagtagctgatgaactcggtggcggttcgggaacgtgctctgtaa      420
TMI I I
I V I S I D Y Y N M F T S I F T L T M M      160
atagtcatttcaattgactattataaatgttcaccagtatatttcacgttaacatgatg      480

S V D R Y I A V C H P V K A L D F R T P      180
agcgtggaccgatacattgccgtgtgtcaccctcgtaaagcgttggaactccgcacccca      540
TMI V
L K A K K I N V C I W L L S S S V G I S      200
ctcaaggccaagaaatataatgtctgcatctggctgcttctcgtcgtctgtgggaattct      600
      ◆
A I V L G G T K T T E D T T E C A L Q F      220
gcaatagtctcgggaggaacaaaacacagaagataaccacagaatgtgctgcagttc      660
      TMV
P T P Y Q Y W D T L M K V C V F V F A F      240
ccgacaccatatcaatactgggacacccttatgaaggtctgtgtttctcgtctttgccttt      720
      ▲
I I P V F I I T I C Y T L M I L R L K S      260
ataattcctggttttcatacaaatgtgtacacgctgatgattctgctgtttgaagagt      780
      ▲
V R L L S G S R E K D R N L R R I T R L      280
gtgcgtcttctctccggtatcgagaaaaagaccggaatctccgcggatcacaggttg      840

V L V V V A V F I I C W T P I H I F V L      300
gtgcttgggtggcagtttttatcatttgcgtggaccccatccatattttctgctg      900
      TMV I I
V E A L V N V P Q H T A V I S I Y Y V C      320
gttgaggcacttgtaacgtgccacagcacacggcgctcatatccatctactacgtttgc      960

I A L G Y T N S S L N P I L Y A F L D E      340
attgctgtgggttacaccaacagcagcttgaaccaattctgtatgcgttcttggatgaa      1020
      ■
N F K R C F K D F C F P S K I R M E R Q      360
aacttcaagaggtgtttcaagacttctgctttccatctaagataaggatggagcgccaa      1080
      ▲
G N S R V R N T I H D P A Y N K D A D E      380
ggcaacagccgtgtgagaaacaccatccatgaccctgcctacaacaaggatgctgatgaa      1140
      ▲
T N K P V *      385
acgaacaagccggtatga      1158

      ctacgcgtggaaatgtcttctctctccacatgggagggaa      1200
gtgtcgcgaagaccttggttgactcaaataactacagcagtatgaactgcatcgagatgc      1260
agcatgtcttagctgaaactgacatctgtctgcactttaccatattgctgatctgtacata      1320
atcagctttgtaaaaatccgctgcccactatcccaggacaggctagtggaaaaccggac      1380
gtcagacaagcacattttgaaggccagcatttaacttcaaaaggcagaaccagtttagta      1440
ataagctgatgaagaaggtgtatagatatgtatgtatgccacaactgcatttcaaaagt      1500
cagcctacaaaccttgggtgggaggtcgaagcctccgtgtcccaaatccaactgcaaggg      1560
gagaatcggacagccggactagagtgcagaggacctaaactcttttatatagatgtgtc      1620
gttatgtgtgacaatttctgtcatcctctgataatgcatttaacaatactatatatggtt      1680
tcaataaaacccccaaaatagaagcttcacataaaaaaaaaa      1724

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Figure 1 Nucleotide and deduced amino acid sequences for the nKOR cDNA (GenBank accession number AY725197). Within the coding region, putative transmembrane domains (TMI–TMVII) are underlined; an asterisk marks the stop codon. Consensus N-linked glycosylation sites (●) are indicated (symbol above the affected amino acid), as are potential sites for disulfide bond formation (◆), post-translational palmitoylation (■) and phosphorylation (▲).

(Kozak 1989). The open reading frame encodes a protein that is 385 amino acids in length and has an estimated molecular mass of 43 833 Da (in the absence of post-translational modification). BLAST analysis of this amino acid sequence detected seven conserved transmembrane domains and indicated highest sequence identity to G protein-coupled receptors in general, and to mammalian κ opioid receptors specifically. Hydrophobicity analysis also predicted the presence of seven transmembrane domains. Assignment of putative membrane-spanning regions are indicated in Figs 1 and 2, based on results obtained using online protein topology servers (Moller *et al.* 2001), including TMHMM2.0 (Krogh *et al.* 2001) and HMMTOP2.0 (Tusnady & Simon 2001). The predicted protein contains several sites for post-translational modification that are conserved among G protein-coupled receptors, including consensus sites for N-linked glycosylation (asparagine residues in the extracellular N-terminal domain), and amino acid substrates for palmitoylation and phosphorylation within the putative intracellular C-terminal domain. Cysteine residues in the first two putative extracellular loops provide for the formation of a disulfide bond. The sequence Asp-Arg-Tyr (DRY) in the second intracellular loop (immediately following TMIII) is also a feature conserved among members of the G protein-coupled receptor superfamily. The sequence shown in Fig. 1 has been deposited in the GenBank database (Accession AY725197).

Figure 2 compares the deduced amino acid sequence of the nKOR with zebrafish and mammalian κ receptors (sequences from GenBank). The newt receptor is a protein consisting of 385 amino acids, and is larger than that described for mammals (380 amino acids for all four species) or the zebrafish (377 amino acids). This size difference is accounted for mainly by the fact that the extracellular N-terminal domain of nKOR is larger than the corresponding region in the other receptors listed. nKOR shares approximately 75% (74.2–76.0%) amino acid sequence identity with the mammalian receptors, but only 66% identity with the κ opioid receptor cloned from zebrafish. In general, the transmembrane domains, the intracellular loops and the first extracellular loop tend to be conserved among all species listed; the greatest divergences between the newt protein and that of the non-amphibian species occur in the extracellular N-terminal domain, the intracellular C-terminal domain and the second and third extracellular loops (all of which are highly conserved among mammals).

A tree generated by the PHYLIP (v3.6) protein sequence parsimony method demonstrates that the newt amino acid sequence groups with the kappa opioid receptors, as opposed to the three other opioid receptor types (δ , μ and ORL) that have been cloned from other vertebrates (Fig. 3).

Transient expression of nKOR and binding pharmacology

Saturation binding experiments using the κ -selective agonist [3 H]U69593 revealed the presence of a specific, saturable, high-affinity (estimated $K_d=1.514$ nM) binding site on membranes isolated from COS-7 cells transiently expressing nKOR, as shown in Fig. 4. At a dose equivalent to the estimated K_d for [3 H]U69593, specific binding was 95.4% of total binding. Saturation binding data were best fit using a one-site model for receptor–ligand interactions. No specific [3 H]U69593 binding was observed in parallel experiments conducted with membranes isolated from cells that were not transfected. A pharmacological profile of the cloned newt receptor was constructed, based on the ability of various opioid ligands to displace [3 H]U69593 from membranes of COS-7 cells transiently expressing nKOR (Fig. 5 and Table 1). The cloned receptor bound κ -specific ligands with high affinity ($K_i=0.3$ – 6.2 nM); the observed rank order potency was GR89696 > dynorphin A(1–13) > nor-BNI > U69593 > U50488 > BRL52537.

The expressed receptor also demonstrated relatively high affinity for the non-selective opioid receptor antagonist naloxone ($K_i=9.6$ nM) and the ORL-selective ligand nociceptin ($K_i=23.2$ nM). It exhibited lower affinity for the μ -selective agonist DAMGO ($K_i=55.4$ nM) and very low affinity for the δ -selective agonist DPDPE ($K_i=979$ nM).

Binding assays using neuronal P2 membranes

Competition binding experiments using neuronal P2 membranes prepared from *Taricha* brains yielded results very similar to those obtained with the cloned receptor transiently expressed in COS-7 cells (Fig. 6 and Table 1). P2 membrane pellets bound κ -specific ligands with high affinity ($K_i=0.3$ – 5.3 nM); the observed rank order potency for displacing [3 H]U69593 was GR89696 > dynorphin A(1–13) > nor-BNI > U50488 > U69593 > BRL52537.

Discussion

The present investigation documents the cloning, heterologous expression, and pharmacological characterization of a κ opioid receptor that is present in the brain of a urodele amphibian, the rough-skinned newt *T. granulosa*. This represents an extension of previous studies characterizing κ ligand binding in tissue homogenates of whole brains from frogs or toads (Benyhe *et al.* 1994, Newman *et al.* 2002, Simon *et al.* 1982, Simon *et al.* 1984), and purification protocols (Borsodi *et al.* 1986, Simon *et al.* 1987, 1990) providing evidence for distinct κ or κ -like receptors in the amphibian central nervous system.

Extracellular N-Terminus

Taricha granulosa MES-PVQIFREKEQALEAGRLPTCCVPTNSSIWLPLGWHDDYYGLNGSEEAWEGLGHHLNANH
Homo sapiens I GEPGPTC SA.L.P . . A.FP . . AEPDS AGSDA QLEP
Rattus norvegicus I GEPGPTC SA.LLP . . S.FPN.AESDS VGSEDQ QLEP
Cavia porcellus .GR-RR.GPAQPAE.P RNA.LLP.G.A.LP . . AEPDG AGPQDE QLEP
Mus musculus I GDPGPTCS SA.LLP . . S.FPN.AESDS VGSEDQ QLES
Danio rerio .D.NV K.DKCPSSH EK.L . -NFT.QSAVS.I.N-SSIN.S.TT EQ

	TNI	ILI	TMI
<i>Taricha granulosa</i>	TNISPTIPIIIITAVYSMFVVGVLGNSLVMFVIIRYTKMKTATNIIYFNLAMADALVT		TTT
<i>Homo sapiens</i>	AH...A..V.....V.....		.L.....
<i>Rattus norvegicus</i>	AH...A..V.....V.....		.L.....
<i>Cavia porcellus</i>	AH...A..V.....V.....		.L.....
<i>Mus musculus</i>	AH...A..V.....V.....		.L.....
<i>Danio rerio</i>	EAM.L.-.....V.....C.	.Y...H....	.RV.....

	EL1	TMI1	IL2
<i>Taricha granulosa</i>	MPFQSTEYLMNSWPFGNVLC	KIVISIDYNNMFTSIFTLTMMSV	VDRYIACVCHPVKALDFRT
<i>Homo sapiens</i>	. . . V . . . D .		
<i>Rattus norvegicus</i>	. AV . . . D .		
<i>Cavia porcellus</i>	. . V . . D .		
<i>Mus musculus</i>	. . AV . . . D .		
<i>Danio rerio</i>	. . D . L . . E . V .	. VF	. . V

	TMIV										EL2									
<i>Taricha granulosa</i>	PLKAKK	INVC	IWL	SSSV	GV	SA	IV	LG	TKT	TED	T--	T	ECAL	QFP	TPY	QY	WDT	LMK	VCVF	
<i>Homo sapiens</i>		I..	I..						VR..	VDVI	..S	...	DDD	SW..	LF..	I..				
<i>Rattus norvegicus</i>		I..	I..		A..				VR..	VDVI	..S	...	DDE	SW..	LF..	I..				
<i>Cavia porcellus</i>		I..	I..						VR..	VDII	..S	...	DDD	SW..	LF..	I..				
<i>Mus musculus</i>		I..	I..		A..				VR..	VDVI	..S	...	DDE	SW..	LF..	I..				
<i>Danio rerio</i>	M..	I..	IL..	V..	AA..	P..	M..	S..	Q..	NNG..	--T..		D..	V..						

	TMV	IL3	TMVI
<i>Taricha granulosa</i>	VFAFIIPVFIIITICYTLMILRL	KSVRLLSGSREKDRNLRRI	RLVLVVVAVFIIICWTPIH
<i>Homo sapiens</i>	I...V...L..IV.....		VV.....
<i>Rattus norvegicus</i>	...V...L..IV.....		K.....
<i>Cavia porcellus</i>	...V...L..IV.....		
<i>Mus musculus</i>	...V...L..IV.....		K.....
<i>Danio rerio</i>	I.G.VA.LL...V...V...		VV.....

	EL3	TmVII
<i>Taricha granulosa</i>	I F V L V E A L V N - V P Q H T A V I S	I Y Y V C I A L G Y T N S S L N P I L Y A F L D E N F K R C F K D F C F P S K I
<i>Homo sapiens</i>	. . I . . . GS-TSHS . AL S . F R L M	
<i>Rattus norvegicus</i>	. . I . . . GS-TSHS . L S . F V R . . . I M	
<i>Cavia porcellus</i>	. . I . . . GS-TSHS . AL S . F R I M	
<i>Mus musculus</i>	. . I . . . GS-TSHS . AL S . F V R . . . I M	
<i>Danio rerio</i>	. . I . . K . SHG . ET . S . MAA . FF . V R . . . CPGRT	

Intracellular C-Terminus

<i>Taricha granulosa</i>	RMERQGNSRVNTIHD---PAYNKDADETNPV	385		
<i>Homo sapiens</i>	ST.....VQ.----.LR.I.GM....	380	(294)	[76.0%]
<i>Rattus norvegicus</i>	STN.....VQ.----.SMR.VGGM....	380	(290)	[74.7%]
<i>Cavia porcellus</i>	ST.....VQ.----.MRNV.GV....	380	(288)	[74.5%]
<i>Mus musculus</i>	STN.....VQ.----.SMR.VGGM....	380	(288)	[74.2%]
<i>Danio rerio</i>	AGDGR.V....S.LREHTC..EA.NDGQGR..	377	(257)	[66.4%]

Figure 2 The deduced amino acid sequence of nKOR aligned with amino acid sequences of non-amphibian κ opioid receptors. Dots indicate amino acid residues identical to those of *T. granulosa*; dashes indicate sequence gaps. Boxed sequences indicate putative transmembrane domains (TM1–TMVII); putative intracellular loops (IL1–IL3) and extracellular loops (EL1–EL3) are indicated. The total number of residues is listed at the end of each sequence, followed by the number of amino acids identical to the newt (in parentheses) and the overall percent identity (calculated using ClustalW and MVIEW – in brackets). Sequences for species other than *T. granulosa* were obtained from GenBank. Accession numbers: *T. granulosa*, AAU15126; *Homo sapiens*, NP_000903.2; *Rattus norvegicus*, NP_058863.1; *Cavia porcellus*, P41144.1; *Mus musculus*, NP_035141.1; *Danio rerio*, NP_878306.1.

Figure 3 A phylogenetic tree of known full-length opioid receptor amino acid sequences generated by maximum parsimony (PHYLIP 3.6). The tree is rooted in rat somatostatin receptor type 1 (SS1R). Bootstrap values are indicated at the nodes. The newt sequence (boxed) groups with the kappa opioid receptor sequences from other vertebrate classes. ORL, opioid receptor-like; MOR, mu opioid receptor; DOR, delta opioid receptor; KOR, kappa opioid receptor. Sequences for species other than *T. granulosa* were obtained from GenBank. Accession numbers: SS1R *Rattus norvegicus*, P28646; ORL *Danio rerio*, AAN46747.1; ORL *Mus musculus*, P35377; ORL *Rattus norvegicus*, NP_113757.1; ORL *Cavia porcellus*, P47748; ORL *Sus scrofa*, P79292; ORL *Homo sapiens*, NP_872588.1; ORL *Rana pipiens*, AAR08905.1; ORL *Taricha granulosa*, AAU26067.1; MOR *Bos taurus*, NP_776833.1; MOR *Sus scrofa*, Q95247; MOR *Macacca mulatta*, Q9 MYW9; MOR *Homo sapiens*, NP_000905.1; MOR *Rattus norvegicus*, NP_037203.1; MOR *Mus musculus*, P42866; MOR *Taricha granulosa*, AAV28689; NP_035143.1; MOR *Rana pipiens*, AAQ09991.1; MOR *Catostomus commersoni*, CAA71843.1; MOR *Danio rerio*, NP_571782.1; DOR *Danio rerio*, AAP86771.1; DOR *Taricha granulosa*, AAV28690.1; DOR *Rana pipiens*, AAQ09992.1; DOR *Homo sapiens*, NP_000902.2; DOR *Mus musculus*, NP_038650.2; DOR *Rattus norvegicus*, NP_036749.1; KOR *Danio rerio*, NP_878306.1; KOR *Taricha granulosa*, AAU15126; KOR *Cavia porcellus*, P41144.1; KOR *Homo sapiens*, NP_000903.2; KOR *Mus musculus*, NP_035141.1; KOR *Rattus norvegicus*, NP_058863.1.

Figure 4 Saturation analysis of $[^3\text{H}]\text{U69593}$ binding to membranes isolated from COS-7 cells transiently expressing nKOR. Non-specific binding for each ligand dose was determined in the presence of $10\text{ }\mu\text{M}$ nor-BNI. Each data point represents the mean $\pm$ S.E.M. for triplicate measurements. Inset shows Scatchard analysis.

Figure 5 Inhibition of specific $[^3\text{H}]\text{U69593}$ binding to membranes from COS-7 cells transiently expressing nKOR. Approximately 2 nM $[^3\text{H}]\text{U69593}$ was used in all experiments (range = $1.9\text{--}2.9\text{ nM}$); non specific binding was defined as radioactivity remaining bound in the presence of $10\text{ }\mu\text{M}$ non-radioactive U69535. Each data point represents the mean $\pm$ S.E.M. for triplicate measurements.

Table 1 Summary of inhibition binding data obtained in experiments designed to assess the ability of various opioid ligands to displace [³H]U69593 from membranes of COS-7 cells transiently expressing nKOR or neuronal P2 membranes prepared from *Taricha* brain

Ligand	nKOR expressed in COS-7 cells		Brain P2 neuronal pellets	
	IC ₅₀ (nM)	K _i (nM)	IC ₅₀ (nM)	K _i (nM)
κ-selective				
GR89696	0.6	0.3	0.7	0.3
Dynorphin A(1–13)	0.9	0.4	4.4	1.9
nor-BNI	4.5	1.8	7.5	3.2
U69593	7.3	3.1	7.6	4.0
U50488	8.2	3.4	7.2	3.3
BRL52537	14.4	6.2	15.3	5.3
Non-selective				
Naloxone	18.4	9.6	—	—
ORLI-selective				
Nociceptin	67.5	23.2	—	—
μ-selective				
DAMGO	146.8	55.4	—	—
δ-selective				
DPDPE	2725	979	—	—

Starting with total RNA isolated from newt brains, degenerate PCR amplified a cDNA fragment that showed high sequence homology to the mammalian κ receptors. Subsequently, RACE-PCR (using *Taricha*-specific oligo primers) allowed us to extend the original

cDNA sequence in both directions, and culminated in amplification of a contiguous sequence representing a full-length cDNA encoding a protein of 385 amino acids. BLAST analysis of the deduced amino sequence again demonstrated highest sequence identity with known

Figure 6 Inhibition of specific [³H]U69593 binding to neuronal P2 membranes prepared from *Taricha* brains. Approximately 2 nM [³H]U69593 was used in all experiments (range=1.3–2.1 nM); non-specific binding was defined as radioactivity remaining bound in the presence of 10 μM non-radioactive U69593. Each data point represents the mean±S.E.M. for triplicate measurements.

mammalian κ opioid receptors, and maximum parsimony analysis showed that the amino acid sequence clustered with the other vertebrate κ receptors that have been cloned. Analysis of the primary structure of the predicted protein revealed the presence of physical attributes shared by other G protein-coupled receptors that have been identified as opioid receptors. Among these are the presence of seven hydrophobic transmembrane domains, potential sites for N-linked glycosylation in the extracellular N-terminal domain, a conserved DRY motif at the beginning of the second putative intracellular loop, conserved cysteine residues in the second and third extracellular loops, and potential sites for palmitoylation and phosphorylation in intracellular loops as well as the intracellular C-terminal domain (Minami & Satoh 1995). Based on its sequence, we tentatively identified the cDNA we cloned from the brain of *T. granulosa* as the nKOR.

Membranes isolated from COS-7 cells transiently expressing nKOR possessed a saturable, high-affinity ($K_d=1.51$ nM) binding site for the κ -selective agonist [3 H]U69593; no such binding was detected in COS-7 cells that were not transfected. This measurement agrees closely with values reported previously for [3 H]U69593 in studies describing pharmacological profiles of mammalian κ receptors expressed in COS cells, including rat ($K_d=1.46$ nM) (Meng *et al.* 1993), guinea pig ($K_d=1.17$ nM) (Xie *et al.* 1994) and human ($K_d=1.49$ nM) (Simonin *et al.* 1995) κ receptors. The K_d values reported so far for [3 H]U69593 in saturation binding assays employing amphibian brain tissues are slightly higher than our estimate for nKOR: 6.81 nM for the northern grass frog (*R. pipiens*) (Newman *et al.* 2002) and 15.3 nM in the European water frog (*R. esculenta*) (Benyhe *et al.* 1992).

In competition binding experiments designed to assess the affinity of various opioid ligands for the cloned newt receptor, membranes isolated from COS-7 cells transiently expressing nKOR showed high affinity for the κ -selective agonists GR89696, dynorphin A(1–13), U69593, U50488 and BRL52537, as well as the κ -selective antagonist nor-BNI and the non-selective antagonist naloxone. In mammals, high affinity for both U69593 and U50488 is considered one means of pharmacologically distinguishing κ receptors from the other opioid receptor classes, since these agonists bind potently and specifically to κ receptors (Zukin *et al.* 1988, Clark *et al.* 1989) but do not bind to either δ or μ receptors (Raynor *et al.* 1996).

Competition binding assays conducted with neuronal P2 membranes prepared from *Taricha* brains revealed rank order potencies and affinity constants for κ ligands that matched closely the results of experiments employing membranes isolated from COS-7 cells transiently expressing nKOR. All together, our results are consistent with the idea that nKOR is, in fact, a

newt κ opioid receptor, and that nKOR and the κ binding site present in whole newt brains are one and the same.

Like its mammalian counterparts, the expressed nKOR demonstrated little, if any, affinity for the δ -selective agonist DPDPE ($K_i=979$ nM). In contrast, the expressed nKOR showed moderate affinity for DAMGO ($K_i=55.4$ nM), unlike the cloned/expressed mammalian κ receptors, none of which demonstrate measurable affinity for this μ -selective agonist (Meng *et al.* 1993, Yasuda *et al.* 1993, Mansson *et al.* 1994, Xie *et al.* 1994, Simonin *et al.* 1995). Interestingly, the expressed nKOR showed moderately high affinity ($K_i=23.2$ nM) for nociceptin (orphanin FQ), an ORL1-selective agonist and the endogenous ligand for the ORL1 receptor (Meunier *et al.* 1995, Reinscheid *et al.* 1995). In mammals, nociceptin displays essentially no affinity for the canonical κ , δ and/or μ opioid receptors (Meunier *et al.* 1995, Reinscheid *et al.* 1995, Meng *et al.* 1996, Pan *et al.* 2002) and, despite a high degree of sequence homology shared among all of the opioid receptor classes, the ORL1 receptor in mammals does not recognize the classic opioid drugs that bind with high affinity to κ , δ and μ opioid receptors (Owens & Akil 2002). Such discrimination is especially interesting when it is appreciated that nociceptin (FGGFTGARK SARKLANQ) bears a significant degree of sequence identity to dynorphin A (YGGFLRRIRPKLKWQDNQ) and belongs to the family of opioid peptides (Danielson *et al.* 2001).

We recently cloned, expressed and characterized an ORL1 receptor from the brain of *Taricha* (nORL1), and found that the expressed nORL1 binds nociceptin and dynorphin A(1–13) with high affinity (Walthers *et al.* 2005). Research into the physiology of mammalian opioid ligand–receptor interactions has supported the idea of a strict functional dichotomy, in which the classic opioids interact with μ , κ and/or δ receptors to produce analgesia and nociceptin–ORL1 interactions act in an opposing fashion (Reinscheid *et al.* 1998, Danielson *et al.* 2001). Our results suggest that, in contrast to mammals, newts have ORL1 and κ opioid receptors that recognize both nociceptin and dynorphin. This apparent lower specificity might reflect a more basal characteristic of opioid receptors.

Prior research shows that, among G protein-coupled receptors, the N-terminal domain and extracellular loops specify receptor–ligand selectivity (Akil *et al.* 1996). Studies involving expression of chimeric opioid receptors, as well as modeling experiments simulating receptor–ligand interactions suggest that the second extracellular loop of the κ receptor may play a crucial role in recognition of endogenous ligands (Wang *et al.* 1994, Raynor *et al.* 1996, Paterlini *et al.* 1997). We found that the N-terminal domain and the second and third extracellular loops (and to a lesser degree the

first extracellular loop) of nKOR diverge from the amino acid sequences reported for the mammalian κ receptors that have been cloned to date. These differences might confer different standards for ligand selectivity and account for the fact that nKOR recognizes DAMGO and nociceptin, whereas mammalian κ opioid receptors do not. Li *et al.* (1996) noted that a valine residue that is highly conserved in the first extracellular loop of all mammalian κ receptors is replaced by a negatively charged glutamate residue in a partial cDNA sequence isolated for a frog κ receptor, and speculated that the amphibian κ receptor might have the capacity to bind nociceptin. It turns out that nKOR also possesses glutamate instead of valine at the position in question, and does bind nociceptin, as predicted.

Multiple, distinct κ receptor subtypes (κ_1 , $\kappa_{1a/b}$, κ_2 , $\kappa_{2a/b}$, κ_3) have been proposed based partly on binding studies revealing multiple sites possessing different ligand affinities, partly on the basis of pharmacological experiments demonstrating selective interactions with various ligands, and partly on the basis of varying anatomical distributions (Zukin *et al.* 1988, Clark *et al.* 1989, Benyhe *et al.* 1990, Rothman *et al.* 1990, Wollemann *et al.* 1993). One group has reported that two transcripts are derived from a single gene encoding the rat κ receptor, demonstrating the potential for multiple κ receptor splice variants (Yakovlev *et al.* 1995). In general, however, molecular cloning studies have failed to identify cDNA sequences that would account for the multiplicity of κ receptor subtypes. Our results do not rule out the possibility that multiple nKOR subtypes and/or splice variants exist in the newt brain; however, we found no evidence for the expression of more than one type of κ receptor. In multiple, redundant, PCR procedures, we amplified a product that appeared as a single band on ethidium bromide-stained gels, and the sequence of the amplified cDNA was always the same. Additionally, we found that the binding affinities and rank order potencies for κ -selective ligands were essentially the same, regardless of whether binding assays were conducted using membranes isolated from COS-7 cells transiently expressing the cloned nKOR or neuronal P2 membranes prepared from whole *Taricha* brains. Our observations are consistent with the notion that only one type of κ opioid receptor mRNA is expressed in newt brains.

Recent studies show that G protein-coupled receptors can function in homo- and hetero-oligomeric complexes (Dean *et al.* 2001, Angers *et al.* 2002, Lee *et al.* 2003); co-expression experiments demonstrate the formation of opioid receptor heterodimers that exhibit novel ligand-binding properties compared with either receptor expressed alone (Jordan & Devi 1999, George *et al.* 2000, Pan *et al.* 2002). For example, stable co-expression of κ and δ opioid receptors results in the formation of

a functional κ - δ heterodimer exhibiting binding properties that match the κ_2 receptor subtype (Jordan & Devi 1999). Whether or not other functional combinations of opioid receptors might explain the apparent diversity of κ receptor subtypes remains unresolved. We have recently cloned full-length cDNA sequences for *Taricha* δ and μ opioid receptors, and it will be interesting to test the hypothesis that co-expression of two or more types of *Taricha* opioid receptors may generate novel binding profiles matching the pharmacology described for one or more of the proposed κ receptor subtypes.

Early studies with rough-skinned newts demonstrated rapid and potent suppression of courtship behavior (amplectic claspings of females) in males subjected to acute stress or treated with the steroid corticosterone (Moore & Miller 1984). Similarly, male newts treated with bremazocine and ethylketocyclazocine, both κ -selective agonists, showed rapid inhibition of amplectic claspings (Deviche & Moore 1987). Subsequently, it was discovered that there is a highly specific, saturable corticosterone-binding site in neuronal membranes isolated from *Taricha* brains (Orchinik *et al.* 1991) that appears to be a G protein-coupled receptor (Orchinik *et al.* 1992). In a series of ligand-binding competition experiments designed to screen various classes of ligands for ability to displace [3 H]corticosterone from neuronal membranes, it was found that some, but not all, κ ligands were effective (Evans *et al.* 2000). Dynorphin A(1–13), U50488, bremazocine and ethylketocyclazocine were able to displace some (up to 70%) of [3 H]corticosterone specific binding. U69593 (a κ -selective agonist) and nor-BNI (a κ -selective antagonist) were found to be ineffective. These results, together with the behavioral data collected previously, were consistent with the hypothesis that the membrane corticosterone receptor in the *Taricha* brain is located on a κ opioid-like receptor (Evans *et al.* 2000). The *Taricha* brain κ receptor reported here binds all of the κ -selective ligands with high affinity, including dynorphin A(1–13), U50488, nor-BNI and U69593. In addition, membranes isolated from COS-7 cells transiently expressing nKOR showed low, or no, specific binding of [3 H]corticosterone (data not shown). We conclude, therefore, that nKOR is not the membrane receptor responsible for binding corticosterone in the *Taricha* brain.

In summary, we have cloned a full-length cDNA encoding a κ opioid receptor from the brain of an amphibian, the rough-skinned newt *T. granulosa*. This receptor is similar to κ receptors cloned previously from other vertebrates, but shows interesting pharmacological differences with respect to ligand-binding profiles. The newt brain κ receptor does not appear to be the corticosterone-binding site described by our laboratory previously, but it remains possible that co-expression of

two or more opioid receptor types (including the κ receptor) may result in the formation of an oligomeric complex possessing novel binding properties.

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