Orphanin FQ: A Neuropeptide That Activates an Opioidlike G Protein–Coupled Receptor

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A heptadecapeptide was identified and purified from porcine brain tissue as a ligand for an orphan heterotrimeric GTP– binding protein (G protein)– coupled receptor (LC132) that is similar in sequence to opioid receptors. This peptide, orphanin FQ, has a primary structure reminiscent of that of opioid peptides. Nanomolar concentrations of orphanin FQ inhibited forskolin-stimulated adenylyl cyclase activity in cells transfected with LC132. This inhibitory activity was not affected by the addition of opioid ligands, nor did the peptide activate opioid receptors. Orphanin FQ bound to its receptor in a saturable manner and with high affinity. When injected intracerebroventricularly into mice, orphanin FQ caused a decrease in locomotor activity but did not induce analgesia in the hot-plate test. However, the peptide produced hyperalgesia in the tail-flick assay. Thus, orphanin FQ may act as a transmitter in the brain by modulating nociceptive and locomotor behavior.

The existence of supergene families (*1*) and the introduction of screening approaches for sequence similarity have yielded numerous complementary DNA (cDNA) sequences with similarity to other members of a given supergene family but with no known biological activity, the so-called orphan proteins (*2*). We searched for a natural ligand for the orphan G protein–coupled receptor LC132, whose salient feature is its sequence similarity to opioid receptors. This receptor does not bind any of the previously identified opioid peptides or ligands (*3*). Because it is similar in sequence to the μ-, δ-, and κ-opioid receptors (~65% in the transmembrane regions), we assumed that LC132 might also bind a peptidergic ligand and be coupled to the same second messenger systems as the opioid receptors (*4*). We therefore decided to monitor the activation of this receptor through the inhibition of forskolin-stimulated adenosine 3′,5′-monophosphate (cAMP) accumulation in transfected cells expressing LC132. Because the LC132 mRNA is expressed in large amounts in the hypothalamus (*3*), we set out to purify its ligand(s) from homogenates of porcine hypothalamus.

Acetic acid extracts of porcine hypothalamic tissue were prepared and processed (*5*). An initial screening of pools of fractions derived from cation-exchange chromatography revealed two consecutive pools that contained adenylyl cyclase inhibitory activity in LC132-transfected cells but not in untransfected cells (Fig. 1A). The corresponding individual fractions were further fractionated through five steps of reversed-phase high-performance liquid chromatography (rpHPLC), and from these fractions an active compound was finally purified to homogeneity (Fig. 1B). This material was analyzed by mass spectrometry and sequenced by Edman degradation and proved to be a peptide with the primary structure FGGFT-GARKSARKL- ANQ (*6*). Final yields of this peptide were ~200 pmol from 4.5 kg of brain tissue (wet weight). Computer data bank searches revealed that this peptide had not been reported, either alone or as part of a larger protein.

We synthesized a peptide of the same sequence and showed it to be identical to the natural peptide in retention behavior (by rpHPLC) and in molecular size (by mass spectrometry) (*7*). The synthetic peptide also potently inhibited forskolin-stimulated cAMP accumulation, with a median effective concentration (EC_{50}) of 1.05 ± 0.21 nM and a maximal effect of ~80% inhibition at 100 nM (Fig. 2A). This EC_{50} is comparable to that of other neuropeptides at their receptors and indicates that this peptide can be a natural ligand for the LC132 receptor. We have named it orphanin FQ to denote its relation to an orphan receptor and to specify it by the termini of its amino acid sequence. Opioid agonists or antagonists did not affect the inhibition of forskolin-stimulated cAMP accumulation induced by orphanin FQ (*8*).

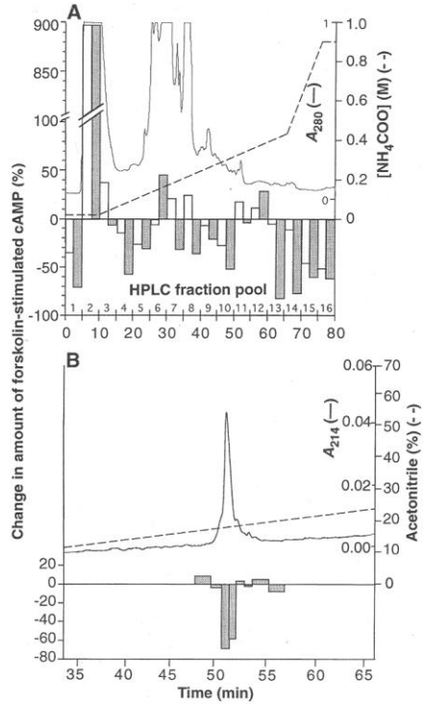

Fig. 1. Purification of orphanin FQ from porcine hypothalamic extracts. (**A**) Cation-exchange HPLC elution profile of the extract, showing the effect of pools of fractions on the amount of forskolin-stimulated cAMP. The extract was prepared as described (*5*), and aliquots of concentrated material were applied to a cation-exchange HPLC column (Protein-Pak SP 8HR, 10 by 100 mm; Waters) equilibrated with 10 mM NH_4COO in 10% (v/v) methanol. The column was eluted with a linear gradient of NH_4COO in 10% methanol at a flow rate of 1 ml/min, and 80 fractions of 1 ml were collected. The 80 fractions were divided into 16 groups of five consecutive fractions, and in each group 10% of each fraction was pooled and lyophilized, yielding 16 pools; 5% of this material was tested in duplicate for its ability to inhibit forskolin-stimulated production of cAMP (*16*). Data were normalized to the amount of cAMP in forskolin-stimulated cells (0%). Pools 13 and 14 showed greater inhibition of forskolin-stimulated adenylyl cyclase activity in transfected cells than in untransfected cells (shaded bars, LC132-transfected CHO cells; open bars, CHO dhfr⁻ wild-type cells). The large increase in cAMP concentration in pool 2 was caused by endogenous cAMP extracted from the tissue. Fractions 60 to 67 contained most of the bioactivity and were pooled for further purification. (**B**) Final rpHPLC. The bioactive material was loaded onto an octyl silica column (Superspher RP select B, 2 by 125 mm; Merck) and eluted with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 0.12 ml/min. Inhibition of forskolin-stimulated adenylyl cyclase activity (*16*) was detected only in the major peak (shaded bars, LC132-transfected CHO cells). During the last two steps of purification, bioassays were only done on cells transfected with LC132 to save material.

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To further analyze the interaction of orphanin FQ with LC132, we developed an ^{125}I-labeled ligand by synthesizing a series of peptide analogs in which tyrosine was substituted at various positions. The Tyr^{14}-substituted peptide and the monoiodo-Tyr^{14} analog were agonists of almost equivalent potency in cAMP assays, with EC_{50} values of 1.02 ± 0.11 nM and 1.78 ± 0.49 nM, respectively (Fig. 2A) (9). The ^{125}I-labeled Tyr^{14}-substituted peptide displayed saturable and displaceable binding to membranes of cells transfected with LC132, with a dissociation constant (K_d) of 0.1 ± 0.02 nM and a maximal binding capacity (B_{max}) of 843 ± 29.7 fmol per milligram of membrane protein (Fig. 2B). These data confirmed that orphanin FQ binds LC132 in a saturable manner and with high affinity.

When the primary structure of orphanin FQ was compared to those of the naturally occurring opioid peptides, three common sequence motifs were found: (i) The NH_2-terminal tetrapeptide FGGF of orphanin FQ is similar to the canonical sequence YGGF of the opioid peptides (Fig. 3). (ii) Two clusters of basic amino acids present in the COOH-terminal portion of orphanin FQ are reminiscent of the arrangement of multiple positively charged residues in dynorphin A (Fig. 3) and β-endorphin. (iii) The COOH-terminal portion of orphanin FQ contains a repetitive sequence (ARKX-ARKXA) that resembles the repeat found at the COOH-terminus of most mammalian β-endorphins (KNAXXKNA) (*10*). However, orphanin FQ activity was not detected at the opioid receptors (*11*), nor did classical opioid peptides bind to the LC132 receptor (*3*). These results therefore suggest a possible evolutionary relation yet a pharmacological divergence between orphanin FQ and the opioid peptides.

We next evaluated the in vivo activity of orphanin FQ in mice after intracerebroventricular (ICV) and intrathecal (IT) administration. The peptide was administered ICV at various doses (0.1 to 10 nmol per 2 μl per mouse; six to eight mice per dose) and the behavior of the mice was observed on a "blind" basis. Locomotor activity was quantified in an automated activity monitor system. At the highest dose, the peptide induced a decrease in horizontal and vertical activity (Fig. 4A) and a decrease in muscular tone in all mice, and it induced ataxia and loss of the righting reflex in 66% of the mice (*12*). At all doses, mice exhibited toe-pinch and tail-pinch reflexes. To examine possible analgesic effects, we conducted a hot-plate test, but no significant inhibition of nociception was detected (Fig. 4B). Only at the highest dose (10 nmol per mouse, ICV) did the animals appear to show an antinociceptive behavior, which was most likely related to the decrease of

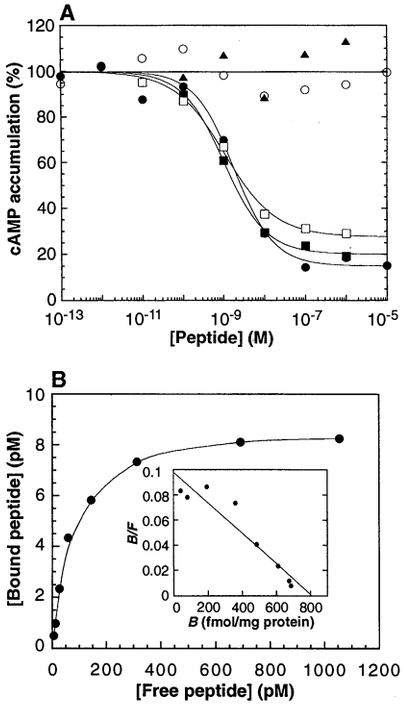

Fig. 2. Pharmacological characterization of orphanin FQ at the LC132 receptor. (**A**) Inhibition of forskolin-stimulated cAMP accumulation in CHO cells transfected with LC132 and treated with orphanin FQ (●), Tyr^{14}– orphanin FQ (■), or monoiodo-Tyr^{14}– orphanin FQ (□). Leu-enkephalin (○) showed no effect. Untransfected CHO cells were treated with orphanin FQ as a control (▲). Data were normalized to the amount of cAMP in forskolin-stimulated cells (100%). Concentrations of cAMP were determined as described (*16*); all incubations were done at least two times in triplicate. Results of a representative experiment are shown. EC_{50} values were determined from fitted curves with Kaleidagraph software. (**B**) Saturation binding of ^{125}I-labeled Tyr^{14}– orphanin FQ. Membranes of LC132-transfected CHO cells were bound to wheat germ agglutinin– coated fluomicrospheres and incubated for 60 min at room temperature with the indicated concentrations of ^{125}I-labeled Tyr^{14}– orphanin FQ (*17*). Nonspecific binding was determined in the presence of 10 nM orphanin FQ. Bound radioligand (*B*) was measured by scintillation proximity. Concentrations of free ligand (*F*) were calculated by subtracting the amount of specific bound ligand from the total amount of radioligand added. The data represent the average of triplicate determinations from two independent experiments. The insert shows a Scatchard transformation of the specific binding; K_d and B_{max} values were calculated with the LIGAND program (*18*).

F G G F T G A R **K S** A R **K L** A N **Q**	**Orphanin FQ**
Y **G G F** L R R I R P K L **K** W D **N Q**	**Dynorphin A**
Y **G G F** M T S E **K S** Q T P **L** V T	**α-Endorphin**
Y **G G F** L R R Q F K V V T	**Dynorphin B**
Y **G G F** L	**Leu-enkephalin**

Fig. 3. Alignment of orphanin FQ with the primary structures of opioid peptides (*6*). Amino acid residues identical to orphanin FQ are shown in bold type.

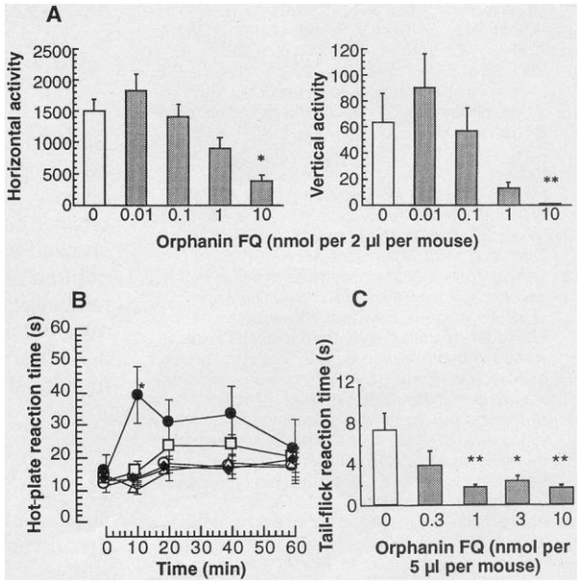

Fig. 4. In vivo activity of orphanin FQ in mice. (**A**) Effect of orphanin FQ on horizontal and vertical locomotor activity as measured in Digiscan Animal Activity Monitors (Omnitech, Columbus, Ohio). Horizontal and vertical activity represents the total number of interruptions of the horizontal and vertical sensors, respectively, during the first 10 min after administration. Groups of six to eight mice were used. (**B**) Effect of orphanin FQ on nociception as measured in the hot-plate test. Groups of six to eight mice were administered phosphate-buffered saline (○) or 0.01 nmol (△), 0.1 nmol (□), 1 nmol (◆), or 10 nmol (●) of peptide per 2 μl per mouse, ICV. Reaction time represents elapsed time until the mice licked their paws. The plate was set at 58°C, and a cutoff time of 60 s was used. (**C**) Hyperalgesic effect of orphanin FQ in the tail-flick test (*19*). Responses were recorded in a Socrel DS 20 apparatus equipped with a 20-V lamp as the heat source. Reaction time represents elapsed time until the mice removed their tails from the heat source. The cutoff time was set to 20 s. Groups of 10 mice were tested 10 min after the administration. Mouse strains used were male MORO, 22 g (A and B) and NMRI, 24 g (C). All data are means ± SEM. Statistical significance was determined by an analysis of variance followed by Dunett's multiple comparison test (A and B) or by Student's *t* test (C); asterisks denote significant differences between the results for mice treated with orphanin FQ and with vehicle (one asterisk, $P < 0.05$; two asterisks, $P < 0.01$).

locomotor activity and muscular tone. Orphanin FQ was also administered IT (2.5 to 10 nmol per 4 μl per mouse), but again no analgesic effect was observed (*13*). We therefore conclude that, in the hot-plate test, orphanin FQ does not induce analgesia. However, when tested in the tail-flick assay, orphanin FQ induced hyperalgesia (0.3 to 10 nmol per 5 μl per mouse, ICV). At a dose of 1 nmol, the reaction time was reduced by ~75% relative to that of vehicle-injected mice (Fig. 4C). The hyperalgesic effect of orphanin FQ was not observed in the hot-plate test, possibly because this test is more dependent on muscular tone and locomotion than is the tail-flick assay (*14*).

Our results demonstrate that despite its structural similarity to the opioid peptides, orphanin FQ appears to be pharmacologically and physiologically distinct from them. Like other neuropeptides, orphanin FQ is presumably synthesized as part of a larger precursor protein (*15*). In view of the increasing number of orphan receptors being identified, it is likely that strategies similar to that used for the identification of orphanin FQ will lead to the description of numerous neurotransmitters or neuropeptides that are unknown today.

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5. Freshly frozen porcine hypothalamic tissue (4.5 kg) was extracted in 9 liters of a solution containing 0.5 M acetic acid, 10 mM ascorbic acid, and 1 mM EDTA. After centrifugation, the supernatant was adsorbed in batches onto C_{18} silica matrix; unbound material was removed by washing and bound material was eluted with 80% methanol. A total of 2 liters of methanolic eluate was concentrated by rotary evaporation to a final volume of 44 ml. Material with a molecular mass of <10 kD was obtained by ultrafiltration and applied in 10 individual runs to a cation-exchange HPLC column.
6. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr; and X, any amino acid.
7. R. K. Reinscheid, unpublished data.
8. Orphanin FQ (10 nM) was applied together with opioid agonists or antagonists (β-endorphin, dynorphin B, dynorphin A 2-13, naloxone, cyclazocine, bremazocine, nor-BNI, ICI 204.448, U 62.066, U 50.488, and U 69.593, all at 1 μM), and the effect on forskolin-stimulated cAMP accumulation in LC132-transfected cells was determined as described (*16*).
9. ^{125}I-labeled Tyr^{14}–orphanin FQ and nonradioactive iodinated peptide were synthesized by the chloramine T method [W. M. Hunter and F. C. Greenwood, *Nature* **194**, 495 (1962)] and purified by reversed-phase HPLC. The monoiodinated species was obtained as a single peak, so that the specific activity was estimated to be 2200 Ci/mmol on the day of synthesis.
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12. Immediately after administration, mice were placed in transparent boxes in groups of three, and behavioral signs were recorded. Emphasis was placed on signs indicative of depressant, stimulant, and autonomic effects [S. Irwin, *Psychopharmacologia* **13**, 222 (1968)]. Animal experimentation and care were done in accordance with the Swiss Federal Ordinance on the Protection of Animals.
13. At 10 nmol of orphanin FQ per mouse IT, all mice tested exhibited hindlimb paralysis and a decrease in locomotor activity (A. Bourson, unpublished data).
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16. CHO dhfr$^-$ cells were transfected by calcium phosphate precipitation with the LC132 cDNA cloned into the eukaryotic expression vector pRcRSV (Invitrogen). Stable clones were selected with G418 and screened for expression of the corresponding mRNA in the reverse transcription polymerase chain reaction. One clone (LC-7) was chosen for further experiments. For determination of concentrations of cAMP, receptor-transfected CHO cells or CHO dhfr$^-$ wild-type cells were plated in 24-well plates and grown to confluency. After removal of the culture medium, portions of HPLC fractions or peptides dissolved in a total volume of 0.2 ml of Dulbecco's modified Eagle's medium [containing 10 mM Hepes (pH 7.4), 1 μM forskolin, and 1 μM phosphodiesterase inhibitor Ro 20-1724] were added and the cells were incubated for 10 min at 37°C. Reactions were stopped by addition of 0.5 ml of ice-cold ethanol and plates were frozen at –80°C for 12 hours. After centrifugation of the plates, portions of the supernatant were removed and dried for cAMP determination. cAMP assays (Biotrak SPA, Amersham) were done according to the manufacturer's instructions.
17. Membranes from LC132-transfected cells (12 to 16 μg of membrane protein per assay) were incubated with various concentrations of ^{125}I-labeled Tyr^{14}–orphanin FQ in a final volume of 0.2 ml of binding buffer [50 mM Hepes (pH 7.4), 10 mM NaCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, 0.1% bovine serum albumin, and 0.025% bacitracin] containing 1 mg of wheat germ agglutinin – coated SPA beads (Amersham). Assays were done in 96-well plates (OptiPlate, Canberra Packard), and the mixtures were agitated for 60 min at room temperature before counting. Under these conditions, membranes become linked to the bead surface through a lectin-glycoprotein interaction. Bound ^{125}I was detected by scintillation proximity [N. Nelson, *Anal. Biochem.* **165**, 287 (1987); N. Bosworth and P. Towers, *Nature* **341**, 167 (1989)] with a TopCount microplate scintillation counter (Canberra Packard). A conversion factor of 6.35 for the ratio of gamma to scintillation counts was obtained by measuring ^{125}I-labeled fluomicrospheres in both counting modes.
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Defects in B Lymphocyte Maturation and T Lymphocyte Activation in Mice Lacking Jak3

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Biochemical studies of signaling mediated by many cytokine and growth factor receptors have implicated members of the Jak family of tyrosine kinases in these pathways. Specifically, Jak3 has been shown to be associated with the interleukin-2 (IL-2) receptor γ chain, a component of the receptors for IL-2, IL-4, IL-7, IL-9, and IL-15. Mice lacking Jak3 showed a severe block in B cell development at the pre-B stage in the bone marrow. In contrast, although the thymuses of these mice were small, T cell maturation progressed relatively normally. In response to mitogenic signals, peripheral T cells in Jak3-deficient mice did not proliferate and secreted small amounts of IL-2. These data demonstrate that Jak3 is critical for the progression of B cell development in the bone marrow and for the functional competence of mature T cells.

B lymphocyte development in the bone marrow and T lymphocyte development in the thymus are dependent on signaling pathways mediated by a complex array of cell surface receptors. These signals include responses to lymphocyte–stromal cell interactions, as well as responses to soluble growth and differentiation factors. The Janus kinase (Jak) family tyrosine kinases, Jak1 and Jak3, have been implicated in signaling through receptors for IL-2, IL-4, IL-7, IL-9, and IL-15, all of which use the IL-2 receptor common γ (γ_c) chain (*1*, *2*). Unlike Jak1, expression of Jak3 is restricted to lymphoid and myeloid cell lines and to hematopoietic tissues such as thymus, bone

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