Identification and Characterization of Two G Protein-coupled Receptors for Neuropeptide FF*

The central nervous system octapeptide, neuropeptide FF (NPFF), is believed to play a role in pain modulation and opioid tolerance. Two G protein-coupled receptors, NPFF1 and NPFF2, were isolated from human and rat central nervous system tissues. NPFF specifically bound to NPFF1 (Kd = 1.13 nM) and NPFF2 (Kd = 0.37 nM), and both receptors were activated by NPFF in a variety of heterologous expression systems. The localization of mRNA and binding sites of these receptors in the dorsal horn of the spinal cord, the lateral hypothalamus, and the thalamic nuclei supports a role for NPFF in pain modulation. Among the receptors with the highest amino acid sequence homology to NPFF1 and NPFF2 are members of the orexin, NPY, and cholecystokinin families, which have been implicated in feeding. These similarities together with the finding that BIBP3226, an anorexigenic Y1 receptor ligand, also binds to NPFF1 suggest a potential role for NPFF1 in feeding. The identification of NPFF1 and NPFF2 will help delineate their roles in these and other physiological functions.

The octapeptide neuropeptide FF (NPFF\(^1\) or F-8-F-amide) and the related octadecapeptide neuropeptide AF (NPAPF or A-18-F-amide) were originally isolated from bovine brain (1) and later determined to be encoded by the same gene and cleaved from a common precursor protein (2). There is a large body of evidence suggesting that NPFF is involved in nociception and in the modulation of opiate-induced analgesia, morphine tolerance, and morphine abstinence (3–11). Interestingly, NPFF possesses both anti-opioid and pro-opioid actions in animal models of pain. The intracerebroventricular administration of NPFF reverses morphine-induced analgesia in rats, and administration of anti-NPFF antibodies increases opiate-induced analgesia (Reviewed in Ref. 12). Conversely, intrathecal administration of NPFF analogs induces a long-lasting, opioid-induced analgesia and potentiates morphine-induced analgesia (12). Other reports have also implicated NPFF in physiological processes such as insulin release, food intake, memory, blood pressure regulation, and electrolyte balance (3).

The exact mechanism underlying the anti- and pro-opioid effects of NPFF is currently unknown, but these seemingly opposing physiological effects could be accounted for by the existence of multiple receptor subtypes. Until now, the cloning of NPFF receptors has remained elusive. NPFF has been shown to activate adenylyl cyclase in mouse olfactory bulb membranes (15), and NPFF binding to rat brain and spinal cord membranes is inhibited by guanine nucleotides (16), suggesting that NPFF elicits its actions through a G protein-coupled receptor (GPCR).

A peptide related to NPFF, FMRF-amide, activates a cation channel (FaNaCh) in the mollusc Helix aspersa (17), which is a member of the DEG/ENaC family of channels. Although an FMRF-amide-gated channel homologous to FaNaCh has not been identified in vertebrates, both FMRF-amide and to a lesser extent, NPFF, can potentiate responses to acid at members of the related ASIC (acid-sensing ion channel) family of acid-sensing channels (34). This action of NPFF is clearly distinct from the effects observed in the CNS which exhibit a considerably higher potency.

Utilizing a GPCR-targeted degenerate PCR methodology, we have identified a novel GPCR that can specifically bind and be activated by neuropeptide FF and the related peptides PQRF-amide and A-18-F-amide, which we have named NPFF1. In addition, we have identified and isolated a second GPCR, structurally related to NPFF1, that can also bind and be activated by NPFF. We have named this second receptor NPFF2. Although NPFF binding sites have been identified in the literature in isolated membranes or in situ, this is the first report identifying a specific receptor system for NPFF.

EXPERIMENTAL PROCEDURES

Materials— NPFF and other commercially available peptides were purchased from Bachem (Torrance, CA). All other peptides and peptides...
were synthesized manually or by using an Advanced Chemtech 396-9000 automated peptide synthesizer (Advanced Chemtech, Louisville, KY). Oligonucleotides were synthesized on an Expedite 8909 oligonucleotide synthesizer (PerkinElmer Life Sciences).

**Degenerate PCR Cloning**—100 ng of rat genomic DNA was subjected to PCR cloning using degenerate primers corresponding to [5'-GNYTWHYBNN- NWSNTGTKTNC-3'] and [5'-AVNADNGBRWAVANNA- NNGGRTT-3'] membrane domains of the rhodopsin GPCR family. Conditions were as follows: 94°C for 3 min; 10 cycles of 94°C for 1 min, 44°C for 1 min, 45 s, and 72°C for 2 min; 30 cycles of 94°C for 1 min, 49°C for 1 min, 45 s, and 72°C for 2 min; 72°C for 4 min. Products were subcloned into the TA cloning kit (Invitrogen, Carlsbad, CA) and sequenced using the ABI Big Dye cycle sequencing protocol and ABI 377 sequencers (Applied Biosystems Inc., Foster City, CA). Nucleotide and amino acid sequence analyses were performed using the Wisconsin Package (GCC, Genetics Computer Group, Madison, WI).

**5'3' Rapid Amplification of cDNA Ends (RACE)**—To determine the full-length coding sequence of AA449919, 5'3' RACE was performed on human spleen Marathon Ready cDNA (CLONTECH, Palo Alto, CA). Nested primers specific to AA449919 were used according to the manufacturer's instructions. The products were sequenced as described above. The Wisconsin Package and Sequencer 3.0 (Gene Codes Corp., Ann Arbor, MI) were used to assemble the full-length contiguous sequence of human NPFF2 (hNPFF2) from the AA449919 EST and the RACE products. The full-length clone was amplified from human spinal cord cDNA using primers flanking the initiating methionine and the stop codon in six independent PCR reactions with the Expand Long Template PCR System (Roche Molecular Biochemicals), and subcloned into pEctNA3.1(+). Each of the six products was fully sequenced, and the construct that agreed 100% with the consensus of the six reactions was used for pharmacological analysis.

**cDNA Library Screening**—Primers specific to the rat receptor fragment were used to isolate a clone representing the full-length BN6 (rNPFF1) receptor from a rat hypothalamic cDNA library (18) using the following PCR protocol: 94°C, hold for 3 min; 40 cycles of 94°C for 1 min, 68°C for 2 min; 4-min hold at 68°C. Positive library pools were subsequently diluted and rescreened by PCR using the same protocol. Positive sub-pools were plated for colony hybridization with 32P-labeled oligonucleotide probes. Isolated positive colonies were chosen, and the respective plasmids were sequenced as described above. Similarly, the full-length hNPFF1 receptor was isolated by PCR screening of pools of human spinal cord cDNA library.

**Electrophysiology**—Chimeric Goqy3, Goqy5, and Goqy_ were generated by PCR using primers encoding human Goq, and the C-terminal five amino acids binding Goq5, and Goq_4. Xenopus oocytes were co-injected with mRNA and injected with mRNA as described (18, 20). Unless otherwise specified, oocytes were voltage clamped at −80 mV. Drugs were applied by superfusion in a solution containing 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, and 5 mM HEPES, pH 7.5.

**Receptor Binding Studies**—Membranes from COS-7 or HEK-293 cells expressing hNPFF1 or hNPFF2 were isolated and subjected to equilibrium binding assays. Solubilized membranes were incubated in binding buffer containing 10 mM LiCl. Each RT-PCR reaction contained 100 ng of total RNA. RNA was quantitated using spectroscopy (A260) and RiboGreen (Molecular Probes) assays. All reagents for RT-PCR (except mRNA and oligonucleotide primers) were obtained from PerkinElmer Life Sciences, and the manufacturer's protocols were used for RT-PCR. Each 96-well plate contained RNA extracted from tissue (in triplicate), controls, and standards and curves to facilitate calculation of the number of NPFF binding sites in human brain.

**mRNA Localization**—For the detection of RNA encoding NPFF receptors, quantitative RT-PCR was performed on mRNA extracted from multiple tissue samples. RNA was prepared using Trizol (Life Technologies, Inc.) or was purchased (CLONTECH). Reverse transcription and PCR reactions were carried out in 50-μl volumes using rtDNA polymerase (PerkinElmer Life Sciences). The following primer sets were synthesized: hNPFF1 forward, 5'-CTGGTGACAGTCACTGTT-3'; hNPFF2 forward, 5'-CCTGGTGGCCGAATGGTCT-3'; rNPFF1 forward, 5'-CTTGTTGGCCGAATGGTCT-3'; rNPFF2 forward, 5'-GAGGACTCTACACCGGTTGGATT-3'; reverse, 5'-GAAGCCCAATCTCGGACCAAT-3'. Fluorogenic probes were synthesized using 6-carboxyfluorescein as the reporter at the 5' end and 6-carboxy-4,7,2',7'-tetramethylrhodamine as the quencher at the 3' end, which were purchased from Applied Biosystems (Foster City, CA). Each RT-PCR reaction contained 100 ng of total RNA. RNA was quantitated using spectroscopy (A260) and RiboGreen (Molecular Probes) assays. All reagents for RT-PCR (except mRNA and oligonucleotide primers) were obtained from PerkinElmer Life Sciences, and the manufacturer's protocols were used for RT-PCR. Each 96-well plate contained RNA extracted from tissue (in triplicate), controls, and standards and curves to facilitate calculation of the number of NPFF binding sites in human brain.
RESULTS

Cloning and Identification of NPFF1—Utilizing a GPCR-targeted degenerate PCR methodology on rat genomic DNA, we identified a novel GPCR fragment most closely related to several peptide-ligand GPCRs. The full-length rat receptor, BN6, was isolated from a rat hypothalamic cDNA library, and the human ortholog, BO102, was subsequently isolated from a human spinal cord cDNA library. Sequence analysis of the rat and human receptors revealed coding sequences of 1296 bp and 1290 bp, and predicted proteins of 432 and 430 amino acids, respectively, which share 87% identity (Fig. 1). Amino acid comparison of BO102 with known GPCRs indicates that it is most similar to human orexin1 (37% identity), human orexin2 (35%), human neuropeptide Y (NPY) Y2 (34%), human cholecystokinin A (CCKA) (34%), human NPY Y1 (32%), mouse GIR (32%), human prolactin-releasing hormone receptor (32%), and human NPY Y4 (31%).

To determine the ligand specificity of this receptor, *Xenopus* oocytes expressing BN6 and a Gαq/z5 chimeric G-protein were used to screen a library of peptidic neurotransmitters. Within this collection only NPFF (1 μM) elicited reliable and robust responses (Fig. 2). Current amplitudes averaged 459 ± 681 nA (n = 13) and these exhibited a concentration dependence with an EC50 of 163 nM (n = 8 oocytes; data not shown). The NPFF-related ligands, A-18-F-amide, Y-18-F-amide, Y-8-F-amide (1 μM) and the C-terminal tetrapeptide PQRF-amide (10 μM), also activated the receptor (data not shown). These results suggested that BN6 encoded a receptor for NPFF, herein called rNPFF1.

Cloning and Identification of NPFF2—A search of Genbank data bases revealed a related human expressed sequence tag (EST) fragment of 532 bp (accession #AA449919), which encoded an amino acid sequence with 59% identity to hNPFF1 and 50% identity to rNPFF1. RACE was used on human spleen cDNA to clone the 5’ and 3’ ends of AA449919, and the full-length receptor, BO89, was amplified from spinal cord cDNA. The rat ortholog of BO89 was cloned by PCR, and named BO119. The coding regions of BO89 and BO119 are 1260 and 1251 bp, encoding proteins with predicted lengths of 420 and 417 amino acids, respectively (Fig. 1). BO89 and BO119 share 78% amino acid identity and are 49–50% identical to rat and human NPFF1.

Oocytes expressing BO89 were robustly activated by NPFF (1 μM, Fig. 2). Mean current amplitudes were 528 ± 99 nA (n = 18). This finding suggested that BO89 was an additional member of the NPFF receptor family; therefore, it was named hNPFF2. Both rNPFF1 and hNPFF2 receptor responses were dependent upon co-expression of the chimeric G-protein Giαq.

Subsequent to the identification of BO89 as NPFF2, a report was published describing the cloning of an orphan receptor sequence named NPGRP (22). NPGRP is nearly identical to NPFF2 except that the N terminus of NPGRP is longer by 102 amino acids. Although it is possible that the more N-terminal initiating methionine could be used for translation of this receptor, the second methionine (the initiating methionine of NPFF2) is surrounded by a good kozak consensus sequence (atcATGaat) and would code for a protein of approximately the
same length as rNPFF2, rNPFF1, and hNPFF1.

**Binding Properties of NPFF1 and NPFF2**—To further assess the pharmacological identity of the human NPFF1 and NPFF2 receptors, the binding properties of the cloned receptors were explored using \[^{125}\text{I}]\text{DMeNPFF} as a radioligand. The specific binding of \[^{125}\text{I}]\text{DMeNPFF} with membranes harvested from COS-7 cells transfected with NPFF1 or NPFF2 receptors at 25 °C reached a maximum by 60 min and remained unchanged for up to 120 min (data not shown). Membranes from transiently transfected COS-7 cells exhibited high affinity, saturable \[^{125}\text{I}]\text{DMeNPFF} binding for both NPFF1 and NPFF2 receptors (Fig. 3, A and B). Nonlinear analysis of \[^{125}\text{I}]\text{DMeNPFF} saturation data yielded an equilibrium dissociation constant (\(K_d\)) of 1.13 ± 0.16 and 0.37 ± 0.03 nM (S.D. \(n = 2\)) for NPFF1 and NPFF2 receptors, respectively (Fig. 3, C and D). Untransfected host cells did not display specific \[^{125}\text{I}]\text{DMeNPFF} binding.

Transient expression of hNPFF1 and hNPFF2 receptors in 293 human embryonic kidney cells (HEK-293 cells) yielded similar \(K_d\) values from saturation studies and a more robust expression (Bmax = 1592 and 510 fmol/mg protein for hNPFF1 and hNPFF2, respectively) as compared with the COS-7 cells (Bmax = 543 and 47 fmol/mg protein for hNPFF1 and hNPFF2, respectively). Therefore, the HEK-293 cells were used to measure the binding affinities (\(pK_d\)) of various NPFF-related peptides in a competition binding assay using \[^{125}\text{I}]\text{DMeNPFF} as the radioligand. The C-terminal RF-amide peptide, PQRF-a-amide, displaced \[^{125}\text{I}]\text{DMeNPFF} binding to both NPFF1 and NPFF2. In addition, NPFF receptors showed high binding affinity for FMRF and lower binding affinity for its \(\alpha\)-Met analog, suggesting that the binding domain of the receptors recognizes the C-terminal RF-amide of NPFF. Other C-terminal RF-amide peptides such as frog PP, an NPY Y4 receptor agonist (23), showed greater affinity for the rat (125-fold) and human (300-fold) NPFF2 receptors compared with the rat NPFF1 receptor (see Table I). Conversely, human PP, human NPY, and peptide YY, which contain a C-terminal RF-amide (24–26), did not bind to either NPFF1 or NPFF2 (data not shown). Interestingly, the synthetic C-terminal RF-amide peptide BIBP3226, an NPY Y1-selective compound (27), displayed 10–60-fold higher affinity for the human and rat NPFF1 receptor as compared with NPFF2 receptors. These findings question the pharmacological selectivity of this peptoid for NPY Y1 receptors, suggesting that BIBP3226 and related compounds may mediate some of their in vivo effects through NPFF receptors rather than through NPY Y1 receptors (28, 29).

**NPFF1 and NPFF2 Coupling to Heterotrimeric G Proteins**—The ability of NPFF1 and NPFF2 receptors to couple functionally to heterotrimeric G proteins was tested using intact COS-7 cells transiently expressing these receptors. NPFF (1 μM) had no effect on either basal or forskolin-stimulated cAMP formation or PI turn-over in untransfected COS-7 cells, indicating that endogenous adenylate cyclase or PI-coupled NPFF receptors are not expressed in untransfected cells. In COS-7 cells transfected with the rat NPFF1 receptor, NPFF elicited a small (2-fold) increase in total inositol phosphate release with an EC\(_{50}\) of 239 nM (Fig. 4A), which most likely reflects a minor activation of this pathway. Pretreatment of cells expressing the rat NPFF1 receptor with 100 ng/ml pertussis toxin (PTX) for 18 h prevented the NPFF-mediated activation of PI turn-over, suggesting that the activation of the phospholipase C pathway in native cells transiently expressing NPFF1 is most likely secondary to the activation of endogenous PTX-sensitive G proteins and not Ga\(_{q/11}\). When NPFF1 was co-expressed with the Ga\(_{q/11}\) chimera, NPFF stimulation resulted in a much more robust inositol phosphate release response, which was not sensitive to PTX treatment, with an EC\(_{50}\) that was left-shifted 2 log units relative to transfection with NPFF1 alone (Fig. 4B). The PTX insensitivity of the response in cells co-expressing NPFF1 and the Ga\(_{q/11}\) chimera suggests that the PI response in cells co-expressing the chimera is mediated by the activation of phospholipase C by the Ga\(_{q}\) domain of the chimera and not secondary to activation of endogenous PTX-sensitive G proteins. In COS-7 cells expressing the human NPFF2 alone, we could not detect a PI turn-over response to NPFF.

To further characterize the functional activity of the receptors, the ability of rNPFF1 and hNPFF2 to stimulate intracellular Ca\(^{2+}\) mobilization when co-expressed with different chimeric G proteins was tested in COS-7 cells. Co-transfection of rat NPFF1 or human NPFF2 receptors with either Ga\(_{q/11}\) or Ga\(_{q/14}\) led, in both cases, to the activation by NPFF of intracellular Ca\(^{2+}\) mobilization in a concentration-dependent manner (Fig. 5). The EC\(_{50}\) values for the NPFF-mediated stimulation of intracellular Ca\(^{2+}\) release were in good agreement with the binding affinities of NPFF at NPFF1 and NPFF2 receptors as measured in equilibrium binding assays. However, when Ga\(_{q/14}\) was co-transfected with NPFF1, the activation of intracellular

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**Fig. 3. Binding of \[^{125}\text{I}]\text{DMeNPFF} to crude membranes of cells expressing human NPFF1 and human NPFF2 receptors.** Saturation isotherm at human NPFF1 (A) and human NPFF2 receptors (B) expressed in Cos-7 cells. Competition analysis for the binding of \(10^{-1}–0.2\) nM \[^{125}\text{I}]\text{DMeNPFF} at hNPFF1 (C) and hNPFF2 receptors (D) expressed in HEK-293 cells. Affinities (IC\(_{50}\) values) obtained for inhibition of radioligand binding were converted to \(K_d\) values using the Cheng-Prussoff equation (39). Mean \(K_d\) values calculated from these studies are summarized in Table I. Binding in the absence of excess unlabeled NPFF (1 μM) represents 100% of control binding. Left panel, hNPFF1 expressing cells. Right panel, hNPFF2 expressing cells. Binding data were analyzed by a nonlinear curve-fitting program (Prism, GraphPad Software, San Diego, CA). The results shown are from three independent experiments.
Identification of a Family of NPFF Receptors

**TABLE I**

Pharmacology of cloned NPFF receptors

Affinities, expressed as $K_i$, were obtained from competition binding data using $[^{125}I]1DMeNPFF$ as a ligand at the cloned human and rat NPFF1 and NPFF2 receptors expressed in HEK-293 cells. Results are means ± S.E. obtained from at least three separate experiments.

|       | Human NPFF1 $K_i$ (nM) | Human NPFF2 $K_i$ (nM) | Rat NPFF1 $K_i$ (nM) | Rat NPFF2 $K_i$ (nM) |
|-------|------------------------|------------------------|----------------------|----------------------|
| (N-Tyr^3(NMe)Phe^5)NPFF | 7.9 ± 1.0 | 3.2 ± 0.6 | 1.6 ± 0.05 | 2.0 ± 0.62 |
| Frog PP | 1259 ± 236 | 40 ± 11 | 3980 ± 820 | 50 ± 2.1 |
| FMRF-amide | 0.79 ± 0.28 | 4.0 ± 0.2 | 2.0 ± 0.05 | 8.0 ± 9.5 |
| n-Met-FMRF-amide | 251 ± 106 | 398 ± 26 | 631 ± 118 | 630 ± 41 |
| A-18-F-amide | 63 ± 14 | 1.3 ± 0.37 | 32 ± 9.0 | 6.3 ± 0.14 |
| PQRF-amide | 40 ± 26 | 25 ± 3.0 | 25 ± 3.0 | 25 ± 0.60 |
| BIBP3226 | 126 ± 11 | 1259 ± 111 | 252 ± 2.0 | 1585 ± 71 |

**FIG. 4.** Activation of receptor-mediated PI responses in COS-7 cells expressing the rat NPFF-1 receptor in the presence and absence of Go$\alpha_{q/z}$ chimera. PI turn-over assays were performed as described under "Experimental Procedures." A, concentration-dependent increases in total inositol phosphate accumulation in COS-7 cells transiently expressing the rat NPFF-1 receptor. Control, nontreated; PTX, incubated with PTX 100 ng/ml for 18 h (open squares). B, NPFF-mediated increase of total inositol phosphate accumulation in COS-7 cells transfected with rat NPFF-1 and Go$\alpha_{q/z}$ Control, nontreated; PTX, incubated with PTX 100 ng/ml for 18 h. Results are expressed as $[^{3}H]$inositol phosphates ($[^{3}H]IPs$) released (dpm/well). These are representative data from two observations giving similar results.

**FIG. 5.** NPFF-stimulated activation of intracellular Ca$^{2+}$ mobilization in COS-7 cell expressing recombinant human NPFF receptors and chimeric G proteins. Changes in intracellular Ca$^{2+}$ mobilization were measured in intact COS-7 cells loaded with Fluo 3 as described under "Experimental Procedures." COS-7 cells were co-transfected with Go$\alpha_{q/z}$, Go$\alpha_{q/z}$5, or Go$\alpha_{q/z}$ alone or in combination with the cDNAs encoding the rat NPFF-1 receptor (A) or the human NPFF2 receptor (B). The intracellular Ca$^{2+}$ response was measured from the maximum fluorescence intensity after the addition of NPFF. Results are expressed as mean ± S.E. of the percentage of $E_{max}$ calculated from the maximum response elicited by NPFF (maximum response – basal response) in cells transfected with a combination of NPFF receptor and Go$\alpha_{q/z}$. The data shown are from two independent experiments performed in duplicate.

Ca$^{2+}$ mobilization by NPFF was right-shifted and displayed a weaker maximal response (Fig. 5A). Furthermore, co-transfection of Go$\alpha_{q/z}$ with NPFF2 did not permit intracellular Ca$^{2+}$ mobilization by NPFF (Fig. 5B). No response was detected in cells expressing Go$\alpha_{q/z}$ or Go$\alpha_{q/z}$ alone. These results would suggest that although NPFF1 can couple to cyclase-stimulatory G proteins, NPFF1 and NPFF2 may couple more efficiently to cyclase-inhibitory G proteins in this heterologous system.

Subsequent functional studies monitoring intracellular Ca$^{2+}$ fluxes with hNPFF1 and hNPFF2 were conducted, using a fluorescence imaging plate reader, with transiently transfected COS-7 cells co-expressing either NPFF1 or NPFF2 and Go$\alpha_{q/z}$. NPFF elicited an increase in intracellular Ca$^{2+}$ when either hNPFF1 or hNPFF2 were transfected, whereas there was no response observed in cells transfected with only the Go$\alpha_{q/z}$ chimera. As shown in Table II, PQRF amide acted as a full agonist in cells expressing either the NPFF1 or NPFF2 receptors. In contrast, only cells expressing hNPFF2, but not hNPFF1, responded with an intracellular Ca$^{2+}$ response to frog PP, suggesting that frog PP is an NPFF2-selective agonist.

**Anatomical Distribution of NPFF1 and NPFF2 in the Rat CNS**—The anatomical distribution of NPFF1 and NPFF2 receptor binding sites was revealed throughout the rat CNS by receptor autoradiography using 0.5 nM $[^{125}I]1DMeNPFF$ and making use of the subtype-selective displacers, frog PP and BIBP3226 (Fig. 6). The distribution of the rNPFF1 and rNPFF2 receptor binding sites correlated well with the reported distribution of NPFF-like immunoreactive neurons and terminals (30). Additionally, the distribution of rNPFF1 and rNPFF2 receptors was concordant with previous reports of the...
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TABLE II

Activation of intracellular calcium mobilization by COS-7 cells expressing human NPFF receptors and Goq/z chimera.

Changes in intracellular Ca\textsuperscript{2+} mobilization were measured in intact COS-7 cells loaded with Fluo 3 as described under “Experimental Procedures.” Potencies are given as means of EC\textsubscript{50} values in nM ± S.E. obtained from at least three separate concentration-response curves using 12 different concentrations of each peptide. Maximum responses produced by peptides are expressed as percent of the maximum response produced by NPFF.

| Compound      | NPFF1 EC\textsubscript{50} | NPFF1 response | NPFF2 EC\textsubscript{50} | NPFF2 response |
|---------------|-----------------------------|----------------|-----------------------------|----------------|
| NPF           | 16 ± 3.0                    | 100            | 2.0 ± 0.10                  | 100            |
| FPP           | >10,000                     | 0              | 199 ± 29                    | 78             |
| PQRF-amide    | 199 ± 8.0                   | 93             | 100 ± 9                     | 94             |

DISCUSSION

Neuropeptide FF has been shown to modulate a variety of physiological processes such as insulin release, blood pressure regulation, food intake, electrolyte balance, nociception, opiate-induced analgesia, and morphine abstinence syndrome (3, 4, 6, 12), although its role in pain modulation is the most well defined (3, 6, 12). Involvement of NPFF in such a diverse array of processes suggests that NPFF may interact with more than one receptor subtype or that stimulation of NPFF receptors in various target tissues triggers different processes that are tissue-dependent. In this study, we have isolated and characterized two NPFF receptor subtypes, NPFF1 and NPFF2, that are capable of binding NPFF and related peptides in the nanomolar and subnanomolar range. The evidence obtained by co-expression of chimeric proteins suggests that NPFF receptors may couple more efficiently to the activation of the adenylate cyclase inhibitory class of G proteins (Goq, Goq, Goq). Unfortunately, receptor-mediated adenylate cyclase inhibition was not...
F-amide may be the endogenous ligand for NPFF1 or NPFF2 in ways between species (33), that a related peptide such as A-18-
there may be significant differences in the central NPFF path-
and mice. Possible explanations for these observations are that
species differences in the localization of NPFF binding in ro-
are not surprising. Dupuy
higher levels than found in rats.
pressed high levels of NPFF2 mRNA, human heart expressed
distribution of NPFF receptor mRNA. Although rat heart ex-
gans also demonstrated substantial species differences in the
dominant role in sensory modulation than NPFF2. Peripheral or-
in the distribution of NPFF1 and NPFF2 between the human
explanation of the existence of other unidentified NPFF receptor
systems (33).

The distribution of rat and human NPFF1 and NPFF2
mRNA as measured by RT-PCR is consistent with a broad
modulatory action in the periphery as well as the CNS (Tables
III and IV). However, there are considerable species differences in the distribution of NPFF1 and NPFF2 between the human and rat. NPFF1 mRNA is more abundant in the human spinal cord, which is in sharp contrast to the rat, where NPFF2 mRNA is more predominant. Although NPFF2 mRNA is detectable in COS-7 cells expressing either NPFF1 or NPFF2 receptors through the endogenous repertoire of G proteins in this cell line, indicating that further work is needed to fully characterize the endogenous signaling characteristics of these receptors. The finding that NPFF1 can couple to Gs in this heterologous expression system may explain the results in the report by Gherardi and Zajac (15) that demonstrates NPFF activation of adenylate cyclase in olfactory bulb membranes. Alternatively, the observations of Gherardi and Zajac may be explained by the possibility of the existence of other NPFF receptors that have yet to be discovered or other NPFF receptor systems (33).

The distribution of rat and human NPFF1 and NPFF2
mRNA levels are expressed as percent of highest expressing tissue: spinal cord for NPFF1 and placenta for NPFF2. Some regions represent tissue obtained from one donor.

**TABLE III**

| Region               | hNPFF1 | hNPFF2 |
|----------------------|--------|--------|
| Amygdala             | 44     | 27     |
| Caudate-putamen      | 19     | 9      |
| Cerebellum           | 20     | Trace  |
| Fetal brain          | 4      | 1      |
| Fetal kidney         | 2      | 3      |
| Fetal liver          | Trace  | 1      |
| Fetal lung           | 6      | Trace  |
| Heart                | Trace  | Trace  |
| Hippocampus          | 45     | 7      |
| Hypothalamus         | 21     | 2      |
| Kidney               | 1      | 1      |
| Liver                | Trace  | Trace  |
| Lung                 | 7      | 1      |
| Pancreas             | Trace  | 1      |
| Pituitary            | 2      | 5      |
| Placenta             | Trace  | 100    |
| Small intestine      | 3      | 4      |
| Spinal cord          | 100    | 1      |
| Spleen               | 8      | 4      |
| Stomach              | 1      | Trace  |
| Skeletal muscle      | 1      | 1      |
| Substantia nigra     | 13     | 1      |
| Thalamus             | 30     | 2      |
| Whole brain          | 21     | 8      |

detectable in COS-7 cells expressing either NPFF1 or NPFF2 receptors through the endogenous repertoire of G proteins in this cell line, indicating that further work is needed to fully characterize the endogenous signaling characteristics of these receptors. The finding that NPFF1 can couple to Gs in this heterologous expression system may explain the results in the report by Gherardi and Zajac (15) that demonstrates NPFF activation of adenylate cyclase in olfactory bulb membranes. Alternatively, the observations of Gherardi and Zajac may be explained by the possibility of the existence of other NPFF receptors that have yet to be discovered or other NPFF receptor systems (33).

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modulatory action in the periphery as well as the CNS (Tables
III and IV). However, there are considerable species differences in the distribution of NPFF1 and NPFF2 between the human and rat. NPFF1 mRNA is more abundant in the human spinal cord, which is in sharp contrast to the rat, where NPFF2 mRNA is more predominant. Although NPFF2 mRNA is more abundant in the human spinal cord, its abundance relative to other regions is much lower than seen in rat; this implies that in the human, the NPFF1 receptor might play a more prominent role in sensory modulation than NPFF2. Peripheral organs also demonstrated substantial species differences in the distribution of NPFF receptor mRNA. Although rat heart expressed high levels of NPFF2 mRNA, human heart expressed little of either NPFF1 or NPFF2 mRNA. The human spleen contained transcripts for both NPFF1 and NPFF2 receptors at higher levels than found in rats. Although the difference in mRNA localization between species complicates the interpretation of the data, these results are not surprising. Dupuy et al. (33) demonstrated significant species differences in the localization of NPFF binding in dentate and lagomorph brain and spinal cord, even between rats and mice. Possible explanations for these observations are that NPFF may have different functions in different species, that there may be significant differences in the central NPFF pathways between species (33), that a related peptide such as A-18-F-amide may be the endogenous ligand for NPFF1 or NPFF2 in certain tissues, or that the relative amounts of NPFF1 and NPFF2 vary between other species in the same manner as they do between human and rat. Of course, there is also the possibility of the existence of other unidentified NPFF receptor subtypes or other NPFF receptor systems. Recently, Askwith et al. (34) demonstrated that neuropeptide FF and FMRF-amide activated proton-gated currents from cultured sensory neurons and heterologously expressed acid-sensing or dorsal root acid-sensing ion channels. This activation, however, required a much higher concentration of NPFF and FMRF-amide (EC50 = 33 μM) than is required for NPFF1 or NPFF2 activation (EC50 = 16 and 2 nM, respectively).

Taken together, the results from the RT-PCR and receptor binding studies using [125I]1DMeNPFF have examined distribution of NPFF1 and NPFF2 mRNA and receptor binding sites in the rat CNS. The anatomical distribution of NPFF-like immunoreactivity, NPFF2 mRNA, and NPFF2 receptor binding sites in various CNS regions such as the dorsal root ganglia, spinal cord, spinal trigeminal, parafascicular, and raphe nuclei, and lateral hypothalamus, supports a role for the NPFF2 receptor in nociception. NPFF has been shown to attenuate the analgesic effects of morphine after intrathecal and intracerebroventricular injection (35), and the localization suggests that this effect may be mediated by the NPFF2 receptor. However, a contribution of NPFF1 in nociception should not be ruled out, especially since NPFF1 mRNA is present in very high levels in

**TABLE IV**

| Tissue                  | rNPFF1 | rNPFF2 |
|-------------------------|--------|--------|
| Adipose                 | 3      | 12     |
| Adrenal cortex          | 3      | 5      |
| Adrenal medulla         | 17     | Trace  |
| Amygdala                | 57     | 42     |
| Aorta                   | 1      | 24     |
| Celiac plexus           | 4      | 12     |
| Cerebellum              | 17     | 10     |
| Cerebral cortex         | 22     | 11     |
| Choroid plexus          | 26     | 30     |
| Colon                   | Trace  | 8      |
| Dorsal root ganglia     | 3      | 38     |
| Duodenum                | Trace  | 5      |
| Heart                   | 3      | 82     |
| Hipocampus              | 20     | 8      |
| Hypothalamus            | 100    | 84     |
| Kidney                  | 1      | 20     |
| Liver                   | 2      | 3      |
| Lung                    | 4      | 16     |
| Medulla                 | 23     | 92     |
| Nucleus accumbens       | 35     | 11     |
| Olfactory bulb          | 41     | 10     |
| Ovary                   | 14     | 12     |
| Pancreas                | Trace  | Trace  |
| Pineal                  | 4      | Trace  |
| Pituitary               | 24     | 34     |
| Retina                  | 14     | 40     |
| Salivary gland          | Trace  | 33     |
| Spinal cord             | 24     | 100    |
| Spleen                  | Trace  | Trace  |
| Stomach                 | Trace  | 14     |
| Skeletal muscle         | Trace  | Trace  |
| Striatum                | 17     | 16     |
| Substantia nigra        | 49     | 67     |
| Testes                  | 45     | 4      |
| Thalamus                | 3      | 15     |
| Thymus                  | Trace  | 12     |
| Trigeminal ganglia      | 16     | 57     |
| Urinary bladder         | Trace  | Trace  |
| Uterus                  | Trace  | Trace  |
| Vas deferens            | Trace  | Trace  |
| Whole brain             | 21     | 24     |
the human spinal cord. Although in the rat there is some discordance between the expression of NPFF1 mRNA and binding sites in the dorsal horn of the spinal cord. The inability to observe NPFF1 binding sites might be explained by limitations of detection of the autoradiographic technique or possibly that NPFF1 receptors are expressed on terminal projections of spinal cord neurons outside of the spinal cord. The localization of mRNA and binding sites for NPFF1 and NPFF2 in various components of the basal ganglia, such as the nucleus accumbens, substantia nigra, and the caudate putamen, suggests that NPFF receptors might play an in the regulation of the central dopaminergic system, albeit and indirect one, because previous studies have shown that NPFF binding sites are not found in dopaminergic cell bodies (36).

Some of the highest levels of NPFF-like immunoreactivity have been observed in the rat hypothalamus (3, 37). The localization of NPFF2 receptor binding sites and NPFF2 mRNA in the hypothalamus, a region involved in catecholaminergic and serotonergic feeding systems, circadian feeding, and spontaneous activity, suggests that NPFF2 may be involved in the regulation of ingestive behavior. NPFF itself has been shown to reduce food intake in rats (38). Likewise, the presence of NPFF1 mRNA in the hypothalamus raises the possibility that it may also have a role in hypothalamic function. The neurotropic Y1 receptor antagonist, BIBP3226, is an NPFF1-selective ligand (Table I) which has been shown to block feeding through a nonspecific mechanism, not secondary to inhibition of Y1 (28). Therefore, it is possible that the inhibition of NPY-induced feeding by BIBP3226 and the inhibition of feeding by NPFF, as demonstrated by Murase et al. (38), are mediated through NPFF1.

In addition to BIBP3226 binding to both NPFF receptors with preferential binding to rat and human NPFF1, frog PP, an NPY Y4 agonist, also binds to both NPFF receptor subtypes, displaying a higher affinity for rat and human NPFF2. Furthermore, the levels of amino acid identity and similarity between the two NPFF receptor subtypes and the four known NPY receptor subtypes are compatible with the idea that all six receptors could belong to the same evolutionary lineage. Finally, the human NPFF2 gene is localized in the vicinity of the Y5-Y1-Y2 gene cluster on chromosome 4q31 and the human NPFF1 gene maps to chromosome 10q21, close to the NPY Y4 gene on 10q11–21. This leads us to believe that the NPFF1 and NPFF2 genes may have been generated by gene duplication of ancestral NPY receptor genes (32).

In conclusion, we have identified and isolated two members of a receptor family for NPFF and NPFF-related hormones. The fact that NPFF1 and NPFF2 mRNA transcripts are not always found in the same tissues may explain the varied effects of NPFF in different tissues, as well as the seemingly paradoxical pro- and anti-opiate effects of NPFF. The discovery and characterization of these two NPFF receptors provide the means of identifying receptor-selective pharmaceutical agents necessary to further probe and understand the physiological roles and potential therapeutic applications of NPFF action.

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Addendum—While this manuscript was under review, a report by Elshourbagy et al. (40) appeared describing the identification of HLWAR77, which corresponds to NPFF2, as a receptor for NPFF and NPAF. Like NPFF2, HLWAR77 does not have the extra 102 amino acids that are present in NPFFR, and Elshourbagy et al. conclude that NPFFR is an abberant variant. In addition, Elshourbagy et al. show that HLWAR77 (our NPFF2) likely couples to the cyclase-inhibitory class of G proteins.