

Gene for Pain Modulatory Neuropeptide NPFF: Induction in Spinal Cord by Noxious Stimuli

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Received July 2, 1998; accepted February 3, 1999

This paper is available online at <http://www.molpharm.org>

ABSTRACT

Neuropeptides FF (NPFF), AF (NPAF), and SF (NPSF) are homologous amidated peptides that were originally identified on the basis of similarity to the molluscan neuropeptide FMRF-amide. They have been hypothesized to have wide-ranging functions in the mammalian central nervous system, including pain modulation, opiate function, cardiovascular regulation, and neuroendocrine function. We have cloned the NPFF gene from human, bovine, rat, and mouse, and show that the precursor mRNA encodes for all three of the biochemically identified peptides (NPFF, NPAF, and NPSF). We demonstrate that NPFF precursor mRNA expression by Northern analysis and map sites of expression by in situ hybridization. We confirm the validity of the in situ hybridization by showing that its distribu-

tion in the brain and spinal cord matches the distribution of NPFF and NPSF immunoreactivity. We go on to show that the mRNA levels (as measured by in situ hybridization) in the spinal cord can be up-regulated by a model for inflammatory pain (carrageenan injection), but not by a model for neuropathic pain (lumbar nerve ligation). Our results confirm the evolutionary conservation of NPFF, NPAF, and NPSF neuropeptide expression in mammalian brain. They also provide a context for the interpretation of the pain-sensitizing effects of injections of these peptides that have been previously reported. Our results support a model for the role of these peptides in pain regulation at the level of the spinal cord.

Neuropeptides FF (NPFF), AF (NPAF), and SF (NPSF) (Yang et al., 1985; Yang and Martin, 1995) are related mammalian neuropeptides that were originally identified by their similarity to the molluscan cardioactive peptide FMRF-amide (Price and Greenberg, 1977). These mammalian neuropeptides have been implicated in pain modulation (Yang et al., 1985; Gouarderes et al., 1993), opiate function (Tang et al., 1984; Malin et al., 1990), cardiovascular regulation (reviewed by Panula et al., 1996), and neuroendocrine function (Majane and Yang, 1990; Majane et al., 1993). The peptides may be involved in hypothalamic regulation of pituitary func-

tions because they are present in the hypothalamo-pituitary system, they decrease during salt-loading, and are deficient in the pituitary gland of vasopressin-deficient rats (Majane and Yang, 1990; Majane et al., 1993). Peripherally administered NPFF raises blood pressure in rats, an effect mediated by both peripheral and central mechanisms (reviewed by Panula et al., 1996).

NPFF has been implicated in sensory systems, most notably pain and morphine analgesia (Yang et al., 1985). Intracerebroventricular NPFF has been reported to induce a vigorous abstinence syndrome in morphine-tolerant rats (Malin et al., 1990). NPFF also has been shown to regulate the density of opioid receptors (Rothman et al., 1991; Goodman et al., 1996) and modulate self-administration of morphine (Goodman, 1995). When administered in the third ventricle, NPFF can attenuate the antinociceptive effects of morphine (Tang et al., 1984), but intrathecal NPFF can produce long-lasting antinociception (Gouarderes et al., 1993). These seemingly contradictory results may reflect multiple roles for NPFF in modulation of pain pathways, including modulation of ascending systems, the three-tiered descending pain con-

Supported by Medical Research Council of Finland, Juselius Foundation and Signal Transduction Program of Åbo Akademi University and Howard Hughes Medical Institute (HHMI). F.S.V. was an Associate and E.B.Z. is an Investigator of HHMI. Portions of this work were previously presented in abbreviated fashion in the following abstracts: Vilim FS and Ziff E (1995) Cloning of the neuropeptide NPFF and NPAF precursor from bovine, rat, mouse, and human. *Neurosci Abstr* 21:760; Panula P, Nieminen M, Aarnisalo AA, Lintunen M, Karhunen T, Vilim FS, Ziff E and Karlstedt K (1996) Expression of neuropeptide FF precursor in rat CNS. *Soc Neurosci Abstr* 22:1557; Aarnisalo AA, Nieminen M, Kontinen V, Vilim FS, Kalso E, Ziff E and Panula P (1997) Expression of NPFF mRNA in carrageenan inflammation in the spinal cord. *Soc Neurosci Abstr* 23:1806.

ABBREVIATIONS: NPFF, neuropeptide FF; NPAF, neuropeptide AF; NPSF, neuropeptide SF; CNS, central nervous system; RACE, rapid amplification of cDNA ends; PCR, polymerase chain reaction; DTT, dithiothreitol; SP, Substance P; PAN, primary afferent nociceptor.

trol system and local circuits in the dorsal horn. Involvement in sensory pain systems, autonomic regulation, and hypothalamic functions is in agreement with the known limited distribution of NPFF immunoreactive neurons in the medullary, hypothalamic, and spinal locations (reviewed by Panula et al., 1996). A specific binding site for NPFF in the central nervous system (CNS) has been described (Allard et al., 1989), which may correspond to a G protein-coupled receptor that mediates NPFF's effects on opioid pharmacology and receptor binding. NPFF may potentiate the effects of morphine and endogenous opioids on primary afferents in the dorsal horn, but exert antiopioid actions on the control of supraspinal descending neurons through interneurons. Indeed, enkephalins modulate not only the ascending sensory pain mechanisms but also all three levels of the descending system, specifically the periaqueductal gray, rostral ventral medulla and dorsal horn of the spinal cord (Basbaum and Fields, 1984). NPFF-immunoreactive nerve terminals and binding sites are also found on all these levels (reviewed by Panula et al., 1996).

To study the function of this neuropeptide further, we sought to clone the precursor mRNA and to examine possible regulation of the gene. Perry et al. (1997) identified an mRNA coding for the NPFF precursor from human testis cDNA, but did not describe the distribution of the mRNA in brain. Here we report the cloning of the NPFF precursor mRNAs from human hypothalamus in addition to bovine, rat, and mouse brainstem and hypothalamus cDNA. We show that the NPFF gene in human, murine, bovine, and rat tissues is highly conserved and encodes polypeptides 113 to 115 amino acids long, which are the precursor to NPFF, NPAF, and NPSF. We analyze the expression of NPFF mRNA in the brain and spinal cord and show that its distribution matches that of the NPFF and NPSF peptide immunoreactivity, thus validating the results of the *in situ* analysis. We provide evidence that the NPFF gene is specifically induced in the dorsal horn by inflammatory pain. We present a model in which activity-dependent control of the NPFF gene contributes to the regulation of pain perception.

Materials and Methods

Cloning. Human and rat genomic libraries were obtained from Stratagene (La Jolla, CA). A Y129 mouse genomic library was a kind gift from Dr. Alex Joyner (New York University, School of Medicine, New York). Bovine genomic DNA was extracted from bovine brain using proteinase K digestion, phenol chloroform extraction, and ethanol precipitation. Bovine, rat, and mouse brains were obtained frozen in liquid nitrogen (Harlan Sprague-Dawley, Indianapolis IN) and processed using acid-phenol to extract total RNA (Chomczynski and Sacchi, 1987). mRNA was isolated from total RNA using chromatography over oligo(dT) cellulose (Fast Track; Invitrogen, Carlsbad, CA). First-strand cDNA was synthesized from 1 μ g of mRNA using avian myeloblastosis virus reverse transcriptase (cDNA cycle kit; Invitrogen). Directional cDNA libraries from bovine brainstem and rat hypothalamus and brainstem mRNA were made with the Uni Zap kit (Stratagene) according to the manufacturer's recommendations. Human rapid amplification of cDNA ends (RACE)-ready hypothalamus cDNA (Marathon) was purchased from Clontech (Palo Alto, CA). We also used single-stranded oligonucleotide ligation onto first-strand bovine and rat cDNA to perform 5' RACE (Amplifinder; Clontech). Rat and bovine NPFF mRNA 5' termini were mapped to genomic sequences by sequencing clones of 5' RACE products, and the mouse 5' terminus was mapped by sequencing clones of reverse

transcription-polymerase chain reaction (PCR) products and by inference from the rat structure. PCR was performed with Amplitaq DNA polymerase on a GeneAmp DNA thermal cycler (Perkin-Elmer, Norwalk CT) using 1 μ M of primers and 200 μ M of each dNTP. PCR products were subcloned into the T/A cloning vector (Invitrogen) and sequenced using dye termination (Perkin-Elmer, Applied Biosystems International, Foster City, CA). In all cases, at least three PCR clones were analyzed to obtain consensus sequences. Northern blots were performed on 20 μ g of total RNA run on 1.5% agarose formaldehyde/3-(*N*-morpholino)propanesulfonic acid gels and proteins were transferred (Turboblotter; Schleicher & Schuell, Keene, NH) using 20 times standard saline phosphate EDTA onto positively charged nylon (Pall BioDyne B; Life Technologies, Gaithersburg, MD). The RNA was UV cross-linked to the membrane (Stratalinker; Stratagene) and probed using random primed (Boehringer Mannheim, Indianapolis IN) [32 P]dCTP (NEN, Boston, MA)-labeled cDNA in QuickHyb (Stratagene).

The published bovine sequence for NPAF (Yang et al., 1985) was used to synthesize degenerate oligonucleotide primers CCICARMG-ITTYGG and CCRAAICKYTGIGG. PCR was performed for 42 cycles at a 55°C annealing temperature on first-strand cDNA from bovine brainstem using these primers, and the predicted 57-bp fragment was gel purified and subcloned. Primers to the intervening sequence were used in RACE from the library to identify the flanking sequences in both directions. To identify the rat mouse and human clones, oligonucleotides complementary to the NPFF coding sequence were used in 3' and 5' RACE. This sequence was chosen because HPLC analysis indicated that NPFF peptides were identical in all four species (Majane et al., 1988). Primers designed to the 5' and 3' region were used in PCR from bovine and human genomic DNA and sequenced to reveal the positions of two introns. The mouse and rat clones were used in genomic library screens to isolate and sequence the genomic DNA for the NPFF and NPAF gene.

In Situ Hybridization. Male Wistar rats (250–300 g) were decapitated, and tissues removed and frozen in dry ice-cooled isopentane (Fluka Chemie AG, Buchs, Switzerland). Coronal sections 20- μ m thick cut from tissues embedded in Tissue Tek (Miles, Inc., Elkhart, IN) were thaw mounted on poly-L-lysine (Sigma, St. Louis MO)-coated glass slides and stored at –70°C until used. High specific activity RNA probes were generated from the full-length NPFF-cDNA cloned in PGEM-3Z using the Riboprobe Combination System kit (Promega, Madison WI) in combination with T7 RNA polymerase (Promega) and [35 S]UTP (ICN, Costa Mesa, CA). Sense and antisense probes were prepared from *Xba*I (Promega) linearized template. Frozen sections were fixed for 10 min in ice-cold 4% paraformaldehyde (J. T. Baker, Deventer, Holland) in PBS (pH 7.4), washed twice for 5 min in PBS at room temperature and once in 2 \times standard saline citrate (SSC) (1 \times SSC: 0.3 M NaCl and 0.15 M sodium citrate, pH 7.0), and illuminated with UV light for 5 min. After ethanol dehydration and chloroform delipidation, the sections were incubated at 50°C for 1 h with prehybridization solution (50% formamide, 0.6 M NaCl, 2.5 \times Denhardt's solution, 1 mM EDTA, 500 μ g/ml type III salmon sperm DNA, 500 μ g/ml type III baker's yeast total RNA (Sigma), 50 μ g/ml baker's yeast transfer RNA (Boehringer Mannheim, Germany), and 10 mM Tris-HCl, pH 7.5). The sections were then hybridized at 50°C for 20 to 24 h with NPFF antisense or sense cRNA probes (10⁷ cpm/ml in 50% formamide, 0.6 M NaCl, 2.5 \times Denhardt's solution, 1 mM EDTA, 10% Na⁺ dextran sulfate (Pharmacia, Uppsala, Sweden), 10 mM dithiothreitol (DTT), 100 μ g/ml type III salmon sperm DNA (Sigma), 50 μ g/ml type III baker's yeast total RNA (Sigma), 50 μ g/ml transfer RNA, and 10 mM Tris-HCl, pH 7.5). After hybridization, the slides were washed twice for 30 min in 2 \times SSC containing 0.3 mM DTT at 56°C, RNase A (Boehringer Mannheim GmbH, Mannheim, Germany)-treated for 30 min at 37°C (20 μ g/ml in 0.5 M NaCl and 0.01 M Tris-HCl, pH 7.5), and washed again twice for 30 min in 2 \times SSC containing 0.3 mM DTT. The final 30-min and 3-h high stringency washes were performed in 0.1 \times SSC containing 0.3 mM DTT, 14 mM β -mercaptoethanol, and 0.005% sodium

pyrophosphate at 56°C. The slides were then allowed to cool to room temperature before dehydration in ethanol (50%, 70%, and absolute ethanol containing 0.3 M ammonium acetate). Slides were then apposed to Kodak BioMax MR film (Eastman Kodak, New Haven, CT) for 2 weeks. Images were acquired with the Adobe Photoshop program and printed to produce pictures. Quantification of autoradiographic films was carried out by digitizing the film images with a computer-based image analysis system (the MCID Program, Imaging Research Inc., Ontario, Canada) and by measuring the gray-scale pixel values from the laminae of the dorsal horn. Relative optic density values indicating expression of specific NPFF mRNA were obtained based on a ^{14}C standard curve with correction for background. Nonparametric ANOVA was applied. After film exposure, sections were dipped in photographic emulsion NTB 2 (diluted 1:1 with distilled water; Eastman Kodak) and exposed for 56 days. After development, representative sections were stained with toluidine blue, and samples were embedded in Permount (Fisher Chemical, Fair Lawn, NJ). NPFF mRNA-expressing neurons were counted from spinal cord L5–6 segments on both sides after carrageenan inflammation. Every third section was included. Background densities were obtained from representative areas outside the positive cell areas. These values were 1.5 to 3.2 grains/100 μm^2 . Neurons with grain densities more than 10 times higher than background values were considered positive. The results were analyzed using the paired *t* test. Analysis of NPFF mRNA was carried out by quantitative *in situ* hybridization because of the low level of expression. This method allows analysis of individual rats without pooling of the samples.

Immunohistochemistry. Immunohistochemistry was performed using specific antisera against the C-termini of the two active peptides, NPFF and NPSF, present in the NPFF precursor. The peptides FLFQPQRF-NH₂ (NPFF) and SLAAPQRF-NH₂ (NPSF) were synthesized by solid-phase chemistry, purified by reverse-phase chromatography, and single peaks were verified using mass spectrometry. Peptides (5 mg of each) were coupled to succinylated keyhole limpet hemocyanin (Sigma) with *l*-ethyl-3,3 (dimethylaminopropyl) carbodiimide (Sigma) and antisera were produced in rabbits. Multiple intradermal injections and complete Freund's adjuvant were used in primary immunization, followed by intradermal injection of the conjugate in incomplete Freund's adjuvant 5 weeks later. Sera were tested for reactivity on nitrocellulose filters and brain sections every 10 days after the second immunization. Normal or colchicine (Sigma)-treated male Wistar rats (250–300 g) were perfused with 4% paraformaldehyde and brain sections were processed for immunofluorescence as described previously. Primary antisera were diluted 1:500 to 1:10000, and preadsorption controls with

several peptides were carried out as described earlier. Permissions for animal experiments were obtained from Departmental Committees for Animal Experiments.

Carrageenan Inflammation. The rats were anesthetized with halothane and carrageenan (Sigma); 0.2 mg in 0.1 ml saline was injected into the palm of the left hindpaw 3 h before the rats were sacrificed. The resulting inflammation was quantified by cutting both hindpaws from the knee joint and weighing immediately after the rats were sacrificed to verify the effect of carrageenan as reported earlier (Kontinen et al., 1997). No blood loss occurred during the procedure. It has been shown that a typical hyperalgesia and allodynia develop in these animals (Kontinen et al., 1997).

Neuropathic Pain Model. The model of Kim and Chung (1992) was used. The animals were anesthetized with halothane, and the left L5–6 spinal nerves were exposed, isolated, and ligated tightly with 6–0 silk thread. Rats that developed significant mechanical allodynia (threshold for paw withdrawal after von Frey hair stimulation with the force of 4.2 g or less) at 2 weeks from the ligation were used. This time point was selected as most relevant because the allodynia was fully developed. A change before this time point might reflect the effect of acute nerve ligation rather than a well-developed neuropathic state. Permissions for animal experiments were obtained from Departmental Committees for Animal Experiments.

Results

Gene and mRNA for NPFF and Deduced Precursor.

We cloned the gene and cDNA encoding the NPFF precursor to reveal the processing and regulation of active NPFF and NPFF's role in sensory and autonomic functions. The organization of the NPFF gene from human, murine, bovine, and rat (Fig. 1) is conserved among the four species, with two introns in the gene, both of which fall in coding regions. The sequences of the first 500 nucleotides of the 5' flanking regions of the rat and mouse genes are conserved greater than 90% and a TATA box precedes the mRNA start point of both genes. The mRNA 5' untranslated region is short, approximately 13 nucleotides long. Despite repeated cloning efforts, no alternatively spliced mRNAs were detected. The structure of the NPFF precursor peptide deduced from human hypothalamus cDNA is identical with the one derived from human testis cDNA that was recently reported (Perry et al., 1997). The mRNAs from the four species range from 600 to

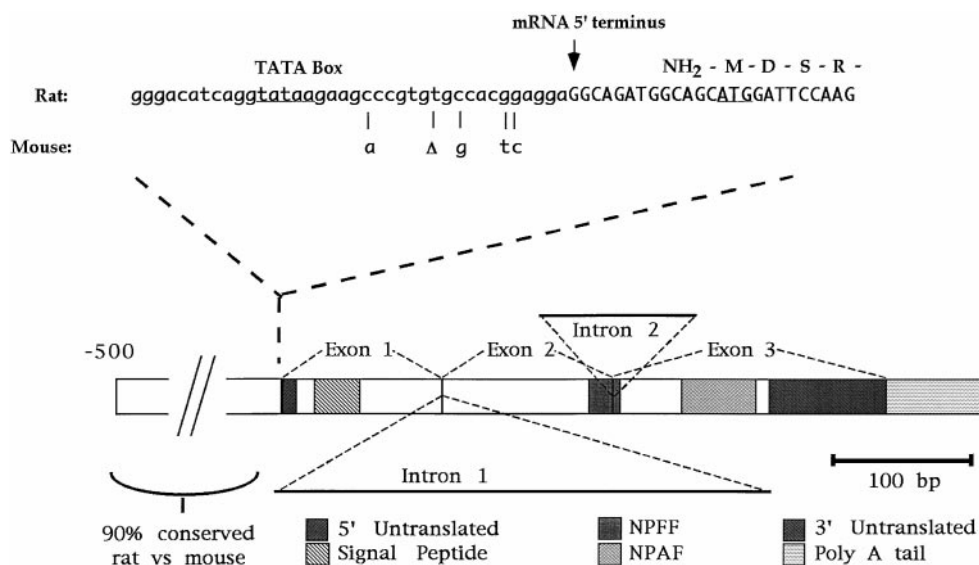


Fig. 1. Organization of NPFF/AF/SF gene and mRNA. Human, rat, mouse, and bovine NPFF/AF/SF genes and mRNAs have a similar organization, with two short introns and three exons. In gene sequence shown at top, 5' flanking region residues are lowercase and residues corresponding to mRNA are uppercase. Differences between rat and mouse gene sequences are indicated. The 500 residues of 5' flanking sequence of mouse and rat genes have over 90% sequence conservation. Position of encoding of mRNA 5' terminus as determined by cDNA cloning is indicated by an arrow, and predicted translation of precursor N terminus is given. A canonical TATA box sequence is found upstream from site of encoding of mRNA 5' terminus. Organization of gene is given below, and locations of exons, introns, and peptide coding sequences are shown.

800 in length and encode highly related precursor polypeptides, 113 to 115 amino acids long (Fig. 2). All contain signal peptide sequences at their N termini and have predicted signal peptide cleavages immediately before glutamate 22 (23 in bovine) (Nielsen et al., 1997). The precursors have overall sequence identities of 40%, with 88% identity between rat and mouse and 71% between bovine and human. Within the sequences of these precursors, we identified structures corresponding to three peptides previously isolated *in vivo*, NPFF, NPAF, and NPSF. Also present for these precursors are C-terminal consensus sequences for peptide processing (reviewed by Eipper et al., 1992), whose cleavage would yield the predicted mature amidated peptides. In addition, the predicted mature peptides all share the C-terminal sequence, PQR-amide, which has been shown to be essential for peptide biological activity (Payza and Yang, 1993). Cleavage at the predicted N-terminal processing sites, however, does not yield termini that correspond as clearly to those reported for the peptides isolated *in vivo* (Yang et al., 1985). There is a conserved processing site three amino acids N-terminal to the amino terminus of the biochemically isolated NPFF. It is possible that other mechanisms besides precursor cleavage are responsible for producing the mature peptide or that the biochemically isolated peptides experienced some degradation. Another potential peptide upstream of NPFF, ending in RP-amide, could be processed from the bovine and human precursors, but is not conserved in mouse or rat.

mRNA and Peptide Expression. We used *in situ* hybridization to characterize the areas and cell types that express NPFF mRNA in the CNS. Expression of NPFF mRNA in normal rat brain was limited to hypothalamus, medulla, and dorsal horn of the spinal cord. Neurons in the paraventricular (Fig. 3) and supraoptic nucleus displayed moderate levels of NPFF mRNA. No mRNA expression was seen in the medial hypothalamus between the dorsomedial and ventromedial nuclei in normal or colchicine-treated animals. A very strong signal was seen in the nucleus of the solitary tract (Fig. 3), especially in its lateral part. A strong signal was also seen in the spinal nucleus of the trigeminal nerve (Fig. 3) and in the dorsal horn of the spinal cord (Fig. 3) at all levels. The signal was limited to the superficial laminae. No NPFF mRNA was found in the posterior pituitary. In peripheral tissues, the adrenal medulla, testis, heart, duodenum, and pancreas displayed no signal. NPFF mRNA was detected by

Northern blotting as a 600-nucleotide species in hypothalamus and brainstem (Fig. 3).

We produced specific antisera against the C-termini of the two active peptides, NPFF and NPSF, present in the NPFF precursor. Identical distributions of immunoreactive cells and fibers was seen with these antisera. Scattered immunoreactive neurons in hypothalamic supraoptic and paraventricular nuclei (Fig. 4) were seen after treatment of the animals with colchicine. A distinct group of immunoreactive neurons was seen in colchicine-treated animals in the medial hypothalamus between the dorsomedial and ventromedial nuclei (Fig. 4) with all antisera against NPFF and NPSF. A distinct population of neurons occupied the nucleus of the solitary tract (Fig. 4), and some cells were also seen in the area postrema. In the spinal cord, both NPFF and NPSF immunoreactivity were limited to nerve fibers and terminals in the superficial laminae (Fig. 4), some fibers in lamina X, and single scattered fibers in a deeper laminae of the dorsal horn. Preadsorption of the NPSF antiserum with the corresponding peptide abolished all reaction (Fig. 4). The posterior pituitary displayed dense NPSF immunoreactive nerve terminals.

Regulation by Inflammatory Pain. To assay for regulation of the NPFF gene by pain-inducing stimuli, we subjected rats to two pain protocols. For one, a neuropathic pain model, L5–6 spinal nerves were ligated. For the second, a noxious irritant model for inflammatory pain, carrageenan, was injected into the left hind paw. The levels of NPFF mRNA in the dorsal horn were determined by *in situ* hybridization and quantitative autoradiography. Fully developed neuropathy was not associated with changes in NPFF mRNA levels, whereas a significantly elevated signal was seen in the ipsilateral dorsal horn on level L4–6 after paw inflammation (Fig. 5). In the experiment displayed in Fig. 5, all sections (25/group) were exposed for autoradiography simultaneously. In other, independent experiments (data from a total of 11 rats), the expression of NPFF mRNA was also significantly higher on the carrageenan side than on the control side ($p = .0037$, data not shown), confirming this result. To elucidate further the nature of the increase in NPFF mRNA in the spinal cord in carrageenan-treated rats, NPFF mRNA expression was analyzed by a second technique. We subjected spinal cord sections to *in situ* hybridization and autoradiography to identify individual cells expressing NPFF mRNA. We then counted the number of cells

Human	MDSRQAAALLVLLLLIDG-GCAEGPGGQQE-DQLSAEEDSEPLPPQDA--	46
Bovine	MDARQAAALLVLLLVTDWSHAEGPGRDGGDQIFMEEDSGAHPAQDA--	48
Rat	MDSK-WAAVLLLLLLLLLRNWGHAEAGSWGGE-DQVFAEEDKGPHPSQYAH	48
Mouse	MDSK-WAALLLLLLLLLLLRNWGHTEAGSWGGE-DQVFAGEDKGPHPPQYAH	48
Human	----QTSGSLLHYLLQAMERPGRSQAFLEFQPORFGRNTQGSWRNEWLSPR	92
Bovine	----QTPRSLLRSLLQAMQRPGRSPAFLFQPORFGRNTRGSWSNKRSLSPR	94
Rat	PDRIQTPGSLMRVLLQAMERPRRNPAFLFQPORFGRNAGWGPSKEQLSPQ	98
Mouse	PDRIQTPGSLFRVLLQAMDTPRRSPAFLFQPORFGRSAWGSWSKEQLNPQ	98
Human	AGEGLNSQFWSLAAPQRFQKK*	113
Bovine	AGEGLSSPFWSLAAPQRFQKK*	115
Rat	ARE-----FWSLAAPQRFQKK*	114
Mouse	AR-----QFWSLAAPQRFQKK*	114

Fig. 2. Species comparison of NPFF/AF/SF peptide precursor. Shaded residues indicate biochemically identified peptides; bovine NPFF (FLFQPQRF-amide), bovine NPAF (AGEGLSSPFWSLAAPQRF-amide), and rat NPSF (SLAAPQRF-amide). C termini are followed by amide donor glycines and consensus processing sites. Putative N-terminal NPFF processing sites (see text) are conserved in all four species. NPAF/NPSF lacks a conserved N-terminal processing site. Arrows denote intron positions, and box region indicates hydrophobic signal peptide.

expressing NPFF mRNA on the treated and control sides of the dorsal horn in each rat. The number of NPFF mRNA-expressing cell profiles (Fig. 5, G and H) in the spinal cord sections was 16.4 ± 0.9 (mean \pm S.E.M.) in the inflammation side and 10.7 ± 0.7 in the contralateral side ($n = 4$, $p = .0029$ in paired t test). Thus, there was an increase following carrageenan treatment of approximately 60% in the number of cells on the treated versus the control side that express NPFF mRNA. This indicates that the increase in NPFF mRNA results from the induction of the mRNA in newly expressing cells of the dorsal horn, or in cells in which the expression level under normal conditions is below the detection limit of the method. The inflammatory pain protocol therefore causes a significant increase in the number of NPFF-expressing neurons, rather than simply an elevation of NPFF expression in neurons that expressed the NPFF gene before carrageenan treatment. The increase in the number of NPFF mRNA containing cells in laminae I-II on the inflammation side as compared with the control side is shown in a representative section in Fig. 6. We conclude that the NPFF gene is induced in intrinsic neurons of the superficial laminae of the dorsal horn by noxious insults associated with carrageenan injection.

Discussion

Cloning of the NPFF gene and mRNA in four species demonstrates that in the bovine, rat, mouse, and human CNS, a single gene encodes the peptide precursor to three related peptides, NPFF, NPAF, and NPSF. Sequencing indicates that the peptides are released from a common precursor polypeptide by N- and C-terminal processing, each poten-

tially involving several known, and potentially some unknown, peptide maturation steps. A similar conclusion for human was reached by cDNA analysis (Perry et al., 1997). We also show that the NPFF gene is induced by inflammatory pain.

Expression of NPFF in Brainstem and Spinal Cord.

In situ hybridization reveals that the nucleus of the solitary tract and dorsal horn of the spinal cord express the highest

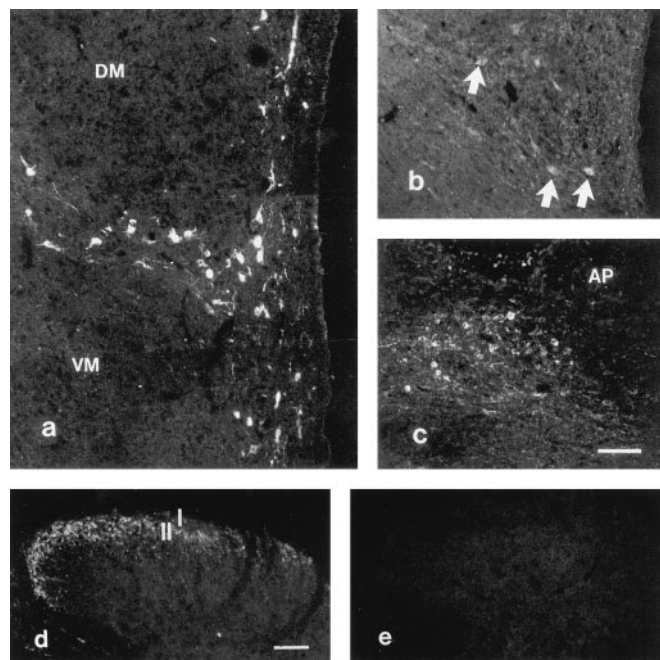


Fig. 4. Immunocytochemical distribution of NPFF and NPSF immunoreactive nerve cells and fibers was similar in CNS. Immunofluorescence with an antiserum against NPSF diluted 1:10000. a, a cell group in hypothalamus between ventromedial (VM) and dorsomedial (DM) nucleus. b, scattered neurons in paraventricular nucleus (arrows). c, numerous neurons in nucleus of solitary tract, next to area postrema (AP). d, a dense network of terminals and fibers in superficial laminae (I and II) of lumbar spinal cord. Antiserum was preadsorbed with $10 \mu\text{M}$ neuropeptide Y, which did not abolish staining. e, A similar section incubated with NPSF antiserum preadsorbed with $5 \mu\text{M}$ NPSF. No reaction is seen. From all areas, NPSF abolished all reaction, NPFF most of it, and FMRF-NH2 had no effect. Unrelated peptides like Met-enkephalin-Arg-Phe or neuropeptide Y did not affect staining pattern or intensity. Scale bar in a-e, $100 \mu\text{m}$.

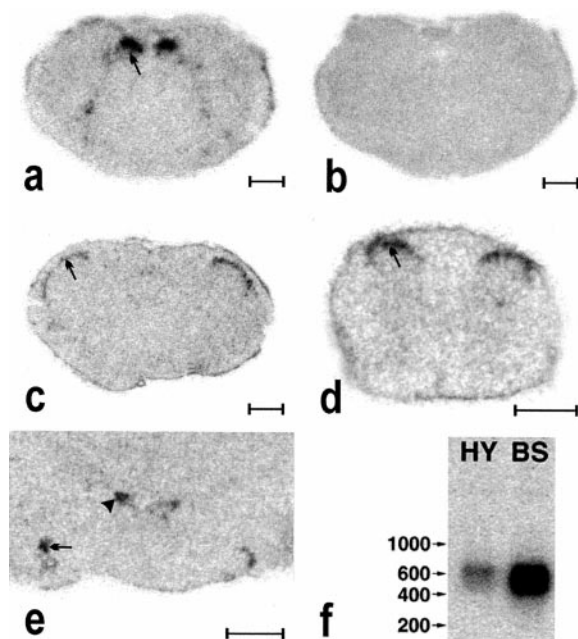


Fig. 3. Expression of NPFF mRNA in rat CNS with ^{35}S -labeled cRNA probes. a, strong expression in nucleus of solitary tract (arrow). b, a consecutive section hybridized with a sense probe shows no signal. c, expression is also evident in spinal nucleus of trigeminal nerve (arrow). d, in spinal cord, expression is limited to superficial dorsal horn (arrow). e, moderate expression can be seen in supraoptic (arrow) and paraventricular (arrowhead) nucleus. f, Northern blot analysis reveals NPFF mRNA as a species of approximately 600 nucleotides in hypothalamus (HY) and brain stem (BS) of rat. Scale bar in a-e, 1 mm.

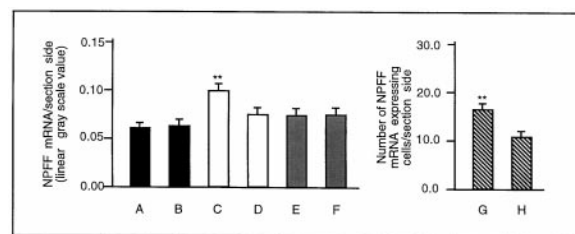


Fig. 5. Expression of NPFF mRNA in rat lumbar spinal cord segments L5-6 is increased ipsilaterally after carrageenan inflammation (column C). ANOVA showed a significant increase on side ipsilateral to inflammation compared with naive control, neuropathic rats and to contralateral side in inflammation group. Values are mean \pm S.E.M., $n = 4$ to 5 animals/group. $*p < 0.01$, nonparametric ANOVA. Black columns (A, B), naive control group; white columns (C, D), inflammation group; hatched columns (E, F), neuropathic group. Columns C and E are ipsilateral to stimulus, D and F are contralateral sides. Columns G and H demonstrate number of NPFF and mRNA-expressing cell profiles in dorsal horn in L5-6 segments in carrageenan-treated rats. G, carrageenan side; H, contralateral side. $*p = 0.0029$ (paired t test), $n = 4$.

levels of NPFF mRNA. NPFF immunoreactivity is also found at these sites (Panula et al., 1996). The moderate expression in the hypothalamic supraoptic and paraventricular nuclei shows that this precursor is expressed in the hypothalamo-pituitary system and gives rise, at least in part, to the NPFF-like peptides detected in this region. Because the number of NPFF- and NPSF-immunoreactive neurons was low even after colchicine treatment, the mature peptides may be rapidly transported to the distal compartments of the neurons. The central hypothalamic cell group between the dorsomedial and ventromedial nucleus apparently also contains NPFF-like peptides (Panula et al., 1996). This group of cells projects to the septum, amygdala, and nucleus of the solitary tract, thus connecting the hypothalamus with the limbic system and medullary autonomic system. However, lack of NPFF precursor mRNA suggests that these cells may express other closely related peptides.

NPFF mRNA and peptide immunoreactivity in the spinal cord localized primarily in the superficial laminae of the dorsal horn. Immunopositive nerve cell bodies are found in this area after local colchicine injections. In contrast, no NPFF-like peptides are found in sensory ganglion cells (Panula et al., 1996) and, as shown here, NPFF mRNA is also absent from the spinal sensory ganglia. Thus, peptides derived from the NPFF precursor are unlikely to be transmitters of the primary afferent nociceptor cell (PAN). The pattern of NPFF precursor expression in rat CNS is distinct from other systems, such as pancreatic polypeptide, neuropeptide Y, and peptide YY (DeQuidt et al., 1990). The pattern associates NPFF expression with sensory transmission in spinal cord, including pain systems, autonomic regulation in the medulla, and neuroendocrine regulation through the hypothalamo-hypophyseal system. These results also suggest that NPFF is not involved in cortical functions and is absent from the cerebellum. Many sites in the CNS that display NPFF mRNA (e.g., dorsal horn of the spinal cord, medulla, and hypothalamus) and receive NPFF-containing projections (Panula et al., 1996), also display binding sites for NPFF (Allard et al., 1992). However, binding sites are also found in cerebral cortex and limbic areas, where little or no NPFF peptide or mRNA has been found. The reasons for this discrepancy are not known, but it is possible that radiolabeled NPFF also binds to receptors for other, hitherto unknown related peptides with e.g., similar C-terminal structure.

NPFF Mechanisms in Dorsal Horn. Intracerebroventricular infusion of NPFF elicits hyperalgesia in normal rats (Yang et al., 1985; Oberling et al., 1993). Hyperalgesia is also a characteristic feature in inflammatory pain models (Hargreaves et al., 1988). However, a long-lasting analgesic effect (Gouarderes et al., 1993) and potentiation of the analgesic effect of morphine (Kontinen and Kalso, 1995) have been found after intrathecal NPFF. Thus, NPFF is a potentially important regulator of pain transmission.

When noxious stimuli activate nociceptors in the periphery, the PAN cells release glutamate and Substance P (SP), transmitters of nociceptive inputs in the dorsal horn. These substances in turn induce the release of NPFF in the spinal cord. This suggests that PAN cells may control spinal NPFF-containing interneurons. The strong expression of NPFF mRNA in cells of the superficial laminae of the dorsal horn seen here suggests that NPFF in the spinal cord is derived from intrinsic neurons (Panula et al., 1996) such as interneurons. Upon release, NPFF may stimulate intrinsic spinal neurons or primary afferent terminals in the dorsal horn, both of which have a high density of NPFF binding sites (Lombard et al., 1995; Gouarderes et al., 1996). The receptor for NPFF and its analogs are reported to reduce the $[Ca^{2+}]_i$ rise that follows depolarization of mouse spinal ganglion cells (Roumy and Zajac, 1996). In so doing, NPFF could antagonize the effects of mobilization of Ca^{2+} by receptors for SP and other neurokinins, thus providing NPFF-induced analgesia. Opioids also inhibit N- and P/Q- type Ca^{2+} channels in target cells (Rhim and Miller, 1994). Modulation of $[Ca^{2+}]_i$ by NPFF and morphine through distinct mechanisms in primary afferent terminals is a possible mechanism for the potentiation of opioid analgesia by NPFF in acute thermal tests in the absence of inflammation (Kontinen et al., 1997).

Induction of NPFF Gene by Inflammatory Pain. There is now clear evidence that carrageenan-induced inflammation induces NPFF expression in ipsilateral spinal cord. We have shown that expression of NPFF mRNA increases significantly, as measured by whole-section autoradiography and by counting NPFF mRNA-expressing cells. NPFF peptide levels also increase in neuronal cell bodies of the ipsilateral spinal cord (Kontinen et al., 1997). In the current study, carrageenan induced a statistically highly significant 1.4-fold increase in NPFF mRNA per section side. Analysis at the cellular level revealed that this increase resulted from a 60% increase in the number of NPFF mRNA

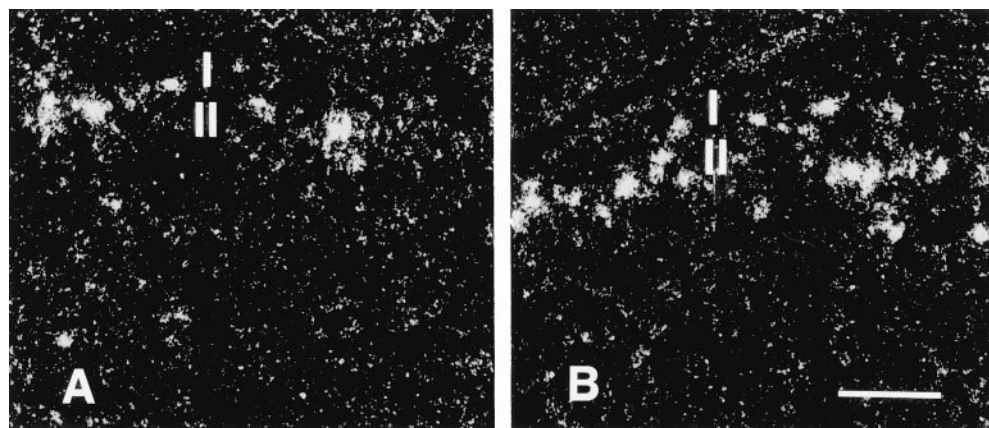


Fig. 6. Increase in number of NPFF mRNA-expressing cells in dorsal horn following carrageenan inflammation. A and B, increased number of NPFF mRNA-containing cells in laminae I-II (I, II) on inflammation side (B) as compared with control side (A) by in situ hybridization in a representative emulsion-coated section. Scale bar, 100 μ m.

expressing cells. Thus, the NPFF promoter is subject to strong activity-dependent regulation by the inflammation protocol in a specific reactive cell population. The induction follows the pattern previously demonstrated for dynorphin, preproenkephalin, and preprotachykinin A expression (Iadarola et al., 1988; Minami et al., 1989; Noguchi et al., 1989; Noguchi and Ruda, 1992). In the carrageenan model of inflammatory pain, both hyperalgesia and increased opioid sensitivity may result from the activation of different classes of nociceptive nerves and an increase in several neuropeptides. Decrease in synthesis of cholecystokinin, a neuropeptide with antiopioid characteristics, following carrageenan inflammation (Dray et al., 1994) may also contribute to hyperalgesia and opioid sensitivity.

The induction of the NPFF gene occurred in a dorsal horn region that contains projection neurons and local circuit neurons that convey nociceptive information. Recently, conditioning electrical stimuli in the rostroventromedial medulla were shown to attenuate noxious heat-evoked, but not mechanically evoked, responses to spinal dorsal horn wide dynamic range neurons in the control (contralateral) side, whereas in the carrageenan-treated side rostroventromedial medulla, stimulation had no effect (Pertovaara et al., 1998). Following direct spinal administration of NPFF, noxious heat-evoked responses, but not mechanically evoked responses, were also attenuated in the carrageenan-treated side. This indicates that NPFF modulates pain responses in carrageenan-treated animals, and may regulate the descending inhibitory pathways. The neuropathic pain model failed to induce the NPFF gene at the time of maximal allodynia, most likely because its pain-inducing mechanisms differ fundamentally from those associated with inflammation. However, we cannot exclude transient changes in NPFF expression during the earlier developmental phases in the neuropathic pain model. The neural injury associated with

ligation prevents the effects of peripheral mediators, including NGF, and leads to a decrease instead of an increase in spinal levels of neurokinins and calcitonin gene-related peptide (Dray et al., 1994). Although no changes in NPFF gene expression were seen in neuropathic rats, NPFF injected directly into the periaqueductal gray produces a significant, naloxone-insensitive attenuation of tactile allodynia in these rats (Wei et al., 1998). This mechanism appears to be at least in part independent of naloxone-sensitive opioid receptors. Sensitization of spinal cord neurons may take place via an interaction of NMDA receptors and SP (Liu et al., 1997). One possible circuitry model is presented in Fig. 7. In this model, damage to tissues and inflammation release pain modulators that activate the sensory C-fibers of PAN cells leading to release of SP and glutamate at synapses with NPFF cells. These cells in turn release NPFF, which exerts a modulatory effect on PAN cell transmission. Sensitization of the PAN cells and release of glutamate and SP may also induce NPFF gene expression in newly expressing cells. Expression of NPFF in a new population of cells may increase the number of PAN cell terminals that are modulated by NPFF. Even if the observed increase in the number of NPFF-expressing cells reflects up-regulation of NPFF production in a cell population that normally expresses the peptide below the detection limit, an increased effect on target cells is possible.

In this manner, regulation of the NPFF gene and NPFF synthesis by inflammatory pain could influence PAN cells transmission and pain perception. A firm mechanism for the antiopioid/pro-opioid effects of NPFF is still difficult to provide. However, the presence of NPFF and its receptors in different crucial sites, including the spinal cord and brain stem (Panula et al., 1996), may explain NPFF's potent site-specific analgesic and hyperalgesic effects. Cloning of the NPFF precursor and gene will facilitate studies of the role of NPFF in pain perception, for example through antisense inactivation of endogenous NPFF and development of NPFF-deficient animal models.

Acknowledgments

We thank A. Joyner for the Y129 mouse genomic library and T. Serra for assistance in preparation of the manuscript.

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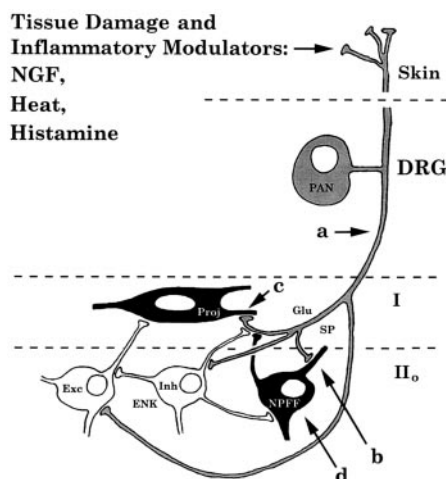


Fig. 7. Proposed circuits and regulation of NPFF neurons in dorsal horn. Tissue damage and inflammation are associated with increased liberation of mediators from immune cells. These activate sensory C-fibers of PAN cells (a), which increase liberation of glutamate and SP at synapses with NPFF cells (b), which release NPFF at synapses with PAN cells, modulating PAN cell transmission (c), and subsequently increase NPFF gene expression in NPFF neuron (d). It is possible that both inhibitory enkephalin-containing neurons and NPFF neurons interact directly with PAN cells, but morphological evidence of this is lacking. Several other important neuropeptides, including dynorphin, neuropeptide Y, and cholecystokinin also modulate neurotransmission and exhibit changes under pathological conditions (Dray et al., 1994; Dubner and Basbaum, 1994).

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