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News and Reviews

Modulatory role of neuropeptide FF system in nociception and opiate analgesia

Hsiu-Ying T. Yang [a,*], Tao Tao [b], Michael J. Iadarola [a]

[a] *Neurobiology and Pain Therapeutics Section, National Institute of Dental and Craniofacial Research, NIH, Building 49, Room 1A07, 49 Convent Drive, MSC 4410, Bethesda, MD 20892-4410, USA*

[b] *Information Resource Branch, National Center for Biotechnology Information, National Institutes of Health, Bethesda, MD, USA*

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Abstract

The tetra-peptide FMRF-NH_2 is a cardioexcitatory peptide in the clam. Using the antibody against this peptide, FMRF-NH_2-like immunoreactive material was detected in mammalian CNS. Subsequently, mammalian FMRF-NH_2 immunoreactive peptides were isolated from bovine brain and characterized to be FLFQPQRF-NH_2 (NPFF) and AGEGLSSPFWSLAAPQRF-NH_2 (NPAF). The genes encoding NPFF precursor proteins and NPFF receptors 1 and 2 are expressed in all vertebrate species examined to date and are highly conserved. Among many biological roles suggested for the NPFF system, the possible modulatory role of NPFF in nocicetion and opiate analgesia has been most widely investigated. Pharmacologically, NPFF-related peptides were found to exhibit analgesia and also potentiate the analgesic activity of opiates when administered intrathecally but attenuate the opiate induced analgesia when administered intracerebroventricularly. RF-NH_2 peptides including NPFF-related peptides were found to delay the rate of acid sensing ion channels (ASIC) desensitization resulting in enhancing acid gated currents, raising the possibility that NPFF also may have a pain modulatory role through ASIC. The genes for NPFF as well as NPFF-R2, preferred receptor for NPFF, are highly unevenly expressed in the rat CNS with the highest levels localized to the superficial layers of the dorsal spinal cord. These two genes are also present in the dorsal root ganglia (DRG), though at low levels in normal rats. NPFF and NPFF-R2 mRNAs were found to be coordinately up-regulated in spinal cord and DRG of rats with peripheral inflammation. In addition, NPFF-R2 immunoreactivity in the primary afferents was increased by peripheral inflammation. The findings from the early studies on the analgesic and morphine modulating activities suggested a role for NPFF in pain modulation and this possibility is further supported by the distribution of NPFF and its receptor and the regulation of the NPFF system *in vivo*.
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Keywords: NPFF; Nociception; Opiate analgesia; RFamide peptides; Asic channels

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* Corresponding author. Tel.: +1 301 402 4981; fax: +1 301 402 0667.
E-mail address: hyang@dir.nidcr.nih.gov (Hsiu-Ying T. Yang).

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1. Introduction

FMRF-NH_2, a cardioexcitatory peptide, was originally isolated from the ganglia of the venerid clam, *Macrocallista nimbosa* (Price and Greenberg, 1977). Subsequently FMRF-NH_2 immunoreactivity was detected in mammalian CNS immunohistochemically using an antiserum raised against FMRF-NH_2 (Boer et al., 1980; Dockray et al., 1983). FMRF-NH_2 shares the same C-terminal tetrapeptide sequence with one of the endogenous opioid peptides, YGGFMRF. In studying pharmacological activity of FMRF-NH_2 and its relationship to enkephalins in perfused clam recta, opposite effects were observed for FMRF-NH_2 and opioid peptides (Greenberg et al., 1983). These observations prompted us to initiate a study on the biochemical structure and physiological functions of endogenous FMRF-NH_2 peptides in mammalian systems especially its relationship to opioid peptides and also its possible modulatory role in pain processing.

The initial biochemical characterization of FMRF-NH_2 immunoreactivity in mammalian CNS has revealed that it is comprised of several peptides, but none of them can be identified as FMRF-NH_2, a conclusion based on chromotagraphic and chemical criteria. It is not surprising, then, that many RF-NH_2 peptides subsequently were identified. To obtain a better tool to study the functional role of FMRF-NH_2 immunoreactivity in mammalian systems, isolation of FMRF-NH_2 immunoreactive peptides was undertaken, and two of them were purified from bovine medulla oblongata and characterized (Yang et al., 1985). They were neuropeptide FF (FLFQPQRF-NH_2) and neuropeptide AF (AGEGLSSPFWSLAAPQRF-NH_2) and initially were referred to as F-8-F-NH_2 and A-18-F-NH_2, mammalian FMRF-NH_2-like peptides, or morphine modulating peptides by various investigators.

Since the isolation and biochemical characterization of neuropeptide FF (NPFF) and neuropeptide AF (NPAF), the functional roles suggested for these peptides, especially for NPFF, include pain modulation (Gouarderes et al., 1993; Panula et al., 1996; Panula et al., 1999; Roumy and Zajac, 1998), water balance (Kalliomaki and Panula, 2004; Majane and Yang, 1991; Sunter et al., 2001), food consumption (Dockray, 2004; Murase et al., 1996; Nicklous and Simansky, 2003; Sunter et al., 2001) with some details to be resolved (Bechtold and Luckman, 2007), modulation of opiate mediated effects (Cesselin, 1995; Harrison et al., 1998; Mollereau et al., 2005b; Panula et al., 1999; Roumy and Zajac, 1998) and cardiovascular actions (Huang et al., 2000; Jhamandas and Mactavish, 2002; Laguzzi et al., 1996).

The cloning of the human NPFF gene was first reported in 1977 (Perry et al., 1997); two N-terminally extended PQRF-NH2 peptides (SQAFLFQPQRF-NH_2 and AGEGLNSQFWSLAAPQRF-NH_2) were predicted from the precursor. NPFF genes for rat, bovine and mouse were identified in 1999 (Vilim et al., 1999) and, again, two N-terminally extended PQRF-NH2 peptides can be predicted from the precursor proteins. The predicted peptides according to cleavage of consensus processing sites for rat and mouse were NPAFLFQPQRF-NH_2 (rNPA-NPFF) and SPAFLFQPQRF-NH_2 (mSPA-NPFF), respectively. NPFF as well as the predicted peptides have all been identified in tissue extracts and the processing of NPAFLFQPQRF-NH_2 and SPAFLFQPQRF-NH_2 to NPFF has been speculated (Bonnard et al., 2003) but still remains unclear and to be determined. Sequence comparisons from recent whole genome sequencing efforts show that the NPFF precursor is highly conserved across many vertebrate species.

High affinity NPFF binding sites were first demonstrated in rat spinal cord and brain using [^{125}I]YLFQPQRF-NH_2 (Allard et al., 1989). This binding site exhibited a regional CNS distribution that was largely dissimilar to that for opioid receptors, although there were some areas of prominent overlap. The binding affinity of [^{125}I]YLFQPQRF-NH_2 to rat spinal cord membranes is not affected by opiates (Allard et al., 1989; Devillers et al., 1994) and, furthermore, NPFF does not show significant affinity for any of the opiate receptor subtypes (Raffa et al., 1994). Subsequently the [^{125}I]YLFQPQRF-NH_2 binding site was demonstrated to be G-protein coupled (Devillers et al., 1994; Payza and Yang, 1993). Identification of NPFF receptor genes was reported by three different groups in the year 2000 and they were referred to as HLWAR77 (Elshourbagy

```
                    1       10        20        30        40        50        60        70        80        90        100       110       120     128
                    |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+-------|
  human|NP_003708.1 MDSRQAAALLVLLLL-IDGGC---AEGPGGQQE-DQLSAEEDSEPLPPQDA---------QTSGSLLHYLLQAMERPGRSQAFLFQPQRFGRNTQGSWR-NEWLSPRAGEGLNSQFWSLAAPQRFGKK
 chimp|XP_001138078.1 MDSRQAAALLVLLLL-IDGGC---AEGPGGQQE-DQLSAEEDSEPLPPQDA---------QTSGSLLHYLLQAMERPGRSQGFLFQPQRFGRNTQGSWR-NERLSPRAGEGLNSQFWSLAAPQRFGKK
monkey|XP_001106733. MDSRQTAALLVLLLL-IDQGC---AEGPGGQQD-HQLPAEEDSEPLLPQDA---------QTSGSLLRYLLQAMERPGRSQAFLFQPQRFGRNTRGSWS-NERLSPRAGEGLNSEFWSLAAPQRFGKK
shrew|AAPY01653874.1 MDSGRAAVLLVVLLL-ADWGL---AKGPGGQDE-DQIFMEEDNGPRPPQDA---------QTPGSLLRSLLQALERPGRSPAFLFQPQRFGRNTRRAWS-NERLSSRAGEGLSSQFWSLAAPQRFGKK
    cow|NP_776548.1 MDARQAAALLLVLLLVTDWSH---AEGPGGRDGGDQIFMEEDSGAHPAQDA---------QTPRSLLRSLLQAMQRPGRSPAFLFQPQRFGRNTRGSWS-NKRLSPRAGEGLSSPFWSLAAPQRFGKK
eleph.|AAGU01070351. MDSRRVAVLLVVLLLVTDWGH---AEGPGGQDEGDQISVEEDKGLHPLQDA---------HTPGSLLRSLLQAMQRPGRSSAFLFQPQRFGRNTRGTWS-NEWLSPRAGEGLSSQFWSLAIPQRFGKK
horse|AAWR01033989.1 MDSRRAAVLLVVLLLITDWGH---TEGPGGQNEGDQMFEEEDTEPYPPQDA---------QTPGPLRRSLLQAMQRPDWSPAFLFQPQRFGRNTWGSWS-TKRLSPRAGEGLNSLFWSLAAPQRFGKK
  cat|AANG01088544.1 MDSRRAALLLLVLLL-TDWGC---AEGPGGQDEGRQIFVEEDSRPRPLQEA---------QTPGSFLHSLLQAMQRPGRSSAFRFQPQRFGRNTRGSWS-SDQLGPRAGEELNAPFWSLAAPQRFGKK
h.hog|AANN01664171.1 MDARRAGVLLLVLLFVTGWGQTDGAEGPEDQDE-DQIISEEDGGPFPLQNA---------QTPGSLLRFLLQAMQRPSRSPAFLFQPQRFGRNSHGSWN-NQRLNPLAGEGLRTQFWNLAAPQRFGKK
    rat|NP_072108.1 MDSKWAAVLLLLLLL-RNWGH---AEEAGSWGE-DQVFAEEDKGPHPSQYAHTPDRI---QTPGSLMRVLLQAMERPRRNPAFLFQPQRFGRNAWGPWS-KEQLSPQARE-----FWSLAAPQRFGKK
  mouse|NP_061257.1 MDSKWAALLLLLLLL-LNWGH---TEEAGSWGE-DQVFAGEDKGPHPPQYAHIPDRI---QTPGSLFRVLLQAMDTPRRSPAFLFQPQRFGRSAWGSWS-KEQLNPQARQ-----FWSLAAPQRFGKK
 z.fish|NP_899189.2 MDAGVWFMLVGVLLVMADQSRSITQDEALEQNKRLLEEDEEQDNMLERPGAAQMNGLLEDRLLVEMLRSLLHGSQRYERNPSVLHQPQRFGRGARSGLSTEERIQSRDWETVPGQIWSMAVPQRFGKK
Tetraodon|BAF34885.1 MDTSVLVSLLALVVAMAGVTGALHIQGSPNKDDLPGSEEQMADSLMEMGGKIADNSN-DNHLLSAVLRALLLGAQRETRN-SVLHQPQRFGRNSNGPTLLDNEMHPHDWEGAPGQIWSMAVPQRFGRK
 Takifugu|BAF34883.1 MDTSALVTLLALVVAMTSVSRALHIQDVLDKEDLPGSEEKMADSMLEMGGRNADNSI-DDYLLNTVLRALLLGAQRETRN-SVLHQPQRFGRNSKGQVMLENEIHPRDWEGAPGQVWSMAVPQRFGRK
          Consensus MD...a..Ll.vll...dwg.....#g...q.e..q...eed..p.p.q.a.........qtpgsllr.LLqam#Rp.RspafLfQPQRFGRn..g.ws..#.l.pra.Eg...qfWs$AaPQRFGkK
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Fig. 1. Annotated and candidate FF-amide precursors are aligned using Multalin with default settings (http://bioinfo.genopole-toulouse.prd.fr/multalin/multalin.html). The headers contain the organism name (shortened if necessary) followed by sequence ID. Some sequence IDs are for wgs contigs, from which the precursors were predicted based on tblastn search and prosplign mapping. The two invariable motifs containing the cleavage sites are highlighted in yellow. Experimentally isolated peptides reported are highlighted in light blue. Note that we were unable to find candidate precursor from dog. Residues in red are identical among the aligned sequences, residues in blue are present in over half of the sequences, and residues in black have no consensus. Dashes represent gaps.

et al., 2000), NPFF receptor1 (NPFF-R1) and NPFF receptor2 (NPFF-R2) (Bonini et al., 2000) and OT7TO22 (Hinuma et al., 2000). Comparison of the amino acid sequences of these genes indicated that NPFF-R2 and NPFF-R1 are identical to the HLWAR77 and OT7TO22 respectively. Both of these receptors are G-protein coupled and show high affinities for NPFF but they are encoded by two different genes. Many studies seem to favor NPFF-R2 as the physiological receptor for NPFF (Bonini et al., 2000; Yang and Iadarola, 2006). NPFF-R2 is coupled to $G_{i/o}$ and activation of this receptor can inhibit the forskolin stimulated adenylate cyclase activity.

Among the several roles suggested for NPFF, the possible involvement of NPFF in modulation of pain and opiate effects has been most widely studied. In this review, the possible modulatory role of NPFF on pain and analgesic effects of opiates will be summarized from several view points: (1) conservation of NPFF and NPFF receptors in various mammalian species, (2) pharmacological effect of NPFF and its analogues, (3) anatomical distribution of NPFF and its receptor, and (4) regulation of the NPFF system in animal models of pain.

2. Whole genome sequencing reveals conservation of NPFF and NPFF receptors

Many whole genome sequencing projects have been undertaken or completed for a wide variety of species. The recent publication of the Macaque genome (Gibbs et al., 2007) prompted us to perform comparative searches for NPFF precursor sequences in order to understand the fundamental nature and extent of expression of this peptidergic system. The curated NCBI RefSeq protein record for human NPFF precursor is NP_003708.1. Using this protein as query and a broad pattern commonly found in this family of proteins, RFG[RK](1,2)-X(25,60)-RFG[RK](1,2), a PHI-BLAST search (http://www.ncbi.nlm.nih.gov/blast/) was performed to identify a list of proteins for use in multiple sequence alignment (Corpet, 1988). To broaden the coverage for organisms with limited protein entries but with a rich collection of whole genome shotgun (wgs) assemblies, we performed tblastn search with this human NPFF precursor to identify contigs with matches and identified putative precursors from those contigs using prosplign (ftp://ftp.ncbi.nih.gov/genomes/TOOLS/ProSplign).

Fig. 1 shows an alignment for the NPFF precursor protein of human, chimpanzee, macaque, North American tree shrew, bovine, elephant, horse, cat, hedgehog, rat, mouse, zebra fish, and two puffer fish, *Takifugu rubripes*, *Tetraodon nigroviridis*. What is evident in the alignment, and quite remarkable, is the conservation of the PQRF-amide sequences within the precursor protein. What has not been determined, except for a limited number of species, is the regional distribution of expression of the precursor. Conserved expression in dorsal spinal cord, one of the regions with the highest expression in the rat, would indicate a critical functional role in nociceptive or somatosensory processes over a broad range of vertebrate species. Indeed, the sequence comparison highlights the conservation and the evolutionary relationships of this system; the latter has been addressed in detail (Osugi et al., 2006).

The conservation also extends to NPFF receptors 1 and 2 as well (Figs. 2 and 3), even though the annotation of them is still a bit unclear. What is clear is that both receptors belong to the seven transmembrane G-protein coupled receptor family which is a very expansive membrane protein receptor family based on olfactory receptors. The characteristic feature is that they all have seven putative trans-membrane domains and this conserved domain (Figs. 2 and 3, highlighted in yellow) is represented by the Pfam record - Pfam00001.13. These data can provide a template to probe receptor function, binding site characteristics, and inter-molecular interactions using mutational analysis of the two receptors.

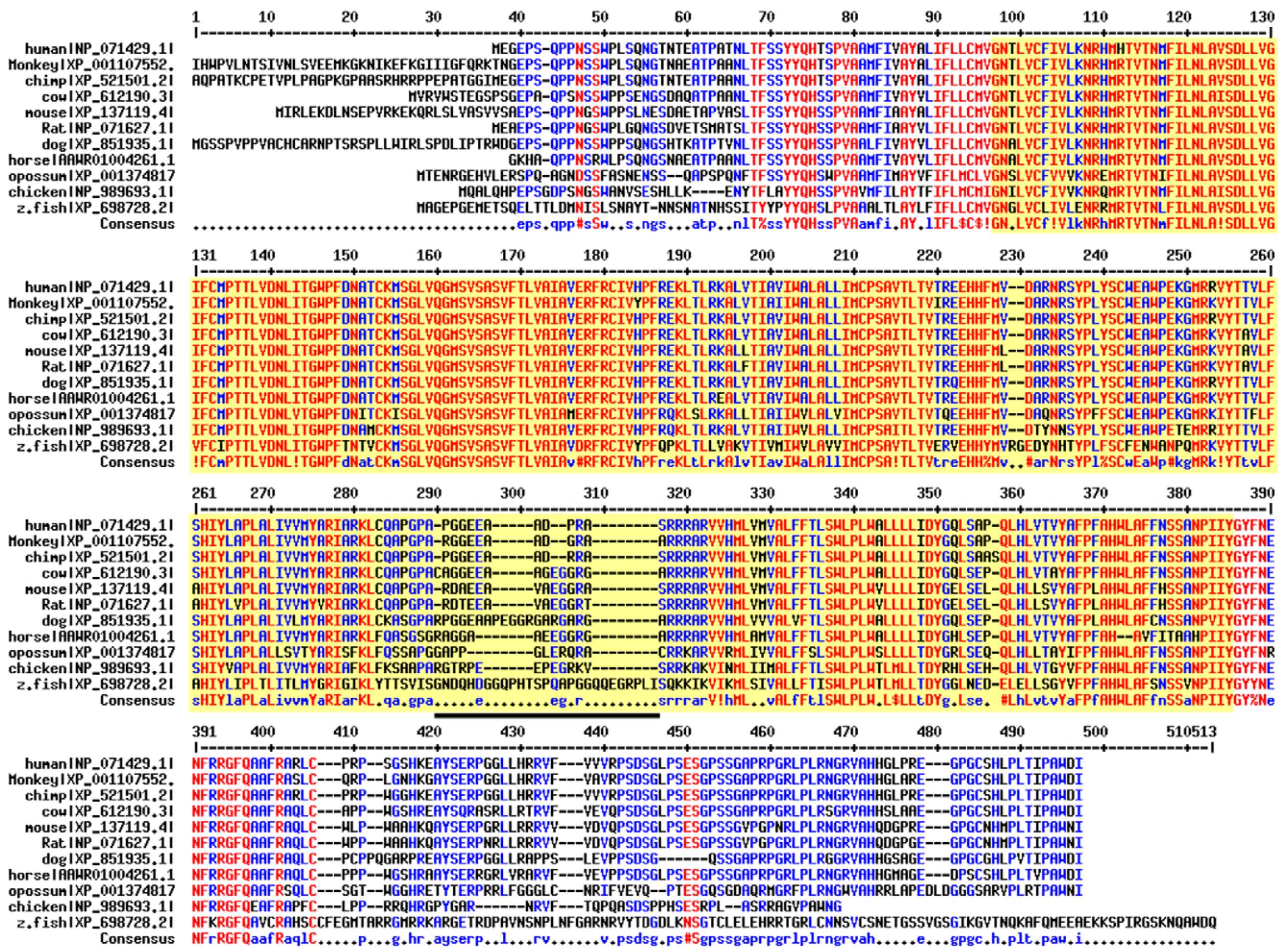

Fig. 2. Annotated and predicted receptors 1 for FF-amide are aligned using Multalin with default settings. The headers contain the organism name (shortened if necessary) followed by sequence ID. For horse, the sequence IDs is for wgs contigs, from which the precursors were predicted based on tblastn search followed by prosplign mapping. The regions mapped to conserved domain, pfam00001.13, are highlighted in yellow. Underline the region is various for unknown reasons. For clarity, the extra long N′-terminal region for chimpanzee and Macaca were not used. This does not affect the overall alignment quality (data not shown).

3. Bioactivities of NPFF-related peptides: (A) pain, morphine modulating activities and receptor binding affinities, and (B) effect on ASIC channels

3.1. Pain and morphine modulating activities and receptor binding affinities

Initially, NPFF and NPAF were isolated from bovine brain and, subsequently, the existence of NPFF in rat and mouse spinal cord and human CSF was determined. In addition to NPFF, other NPFF-related peptides, as shown in Fig. 4, have also been identified in rat, mouse, and human tissues by HPLC followed by RIA or HPLC coupled with mass spectrometry (Bonnard et al., 2001; Bonnard et al., 2003; Burlet-Schiltz et al., 2002; Yang and Iadarola, 2006). We should first address the endogenous NPFF-like peptides that are derived from the NPFF precursor, because different NPFF-related peptides seem to differ in their bioactivities. From the cloned NPFF precursor protein, an N-terminal extended NPFF was readily predicted from the consensus processing sites (Fig. 4). Rat NPA-NPFF (rNPA-NPFF) as predicted from the precursor was detected in a rat spinal cord extract and furthermore the quantity of rNPA-NPFF was determined by RIA to be much more abundant than that of NPFF (Bonnard et al., 2001). Mouse SPA-NPFF (mSPA-NPFF) predicted from the mouse NPFF precursor protein was identified in mouse spinal cord extract, but the quantity seemed to be much lower than that of NPFF (Bonnard et al., 2001; Bonnard et al., 2003). Processing of human proNPFF was studied by using human neuroblastoma and COS cells transfected with human proNPFF cDNA. All three NPFF-related peptides human SQA-NPFF (hSQA-NPFF), NPFF and human NPAF (hNPAF) were identified as the processed products (Bonnard et al., 2003) (Fig. 4). Enzymatic conversion of hSQA-NPFF to NPFF was speculated from the known proteases, but

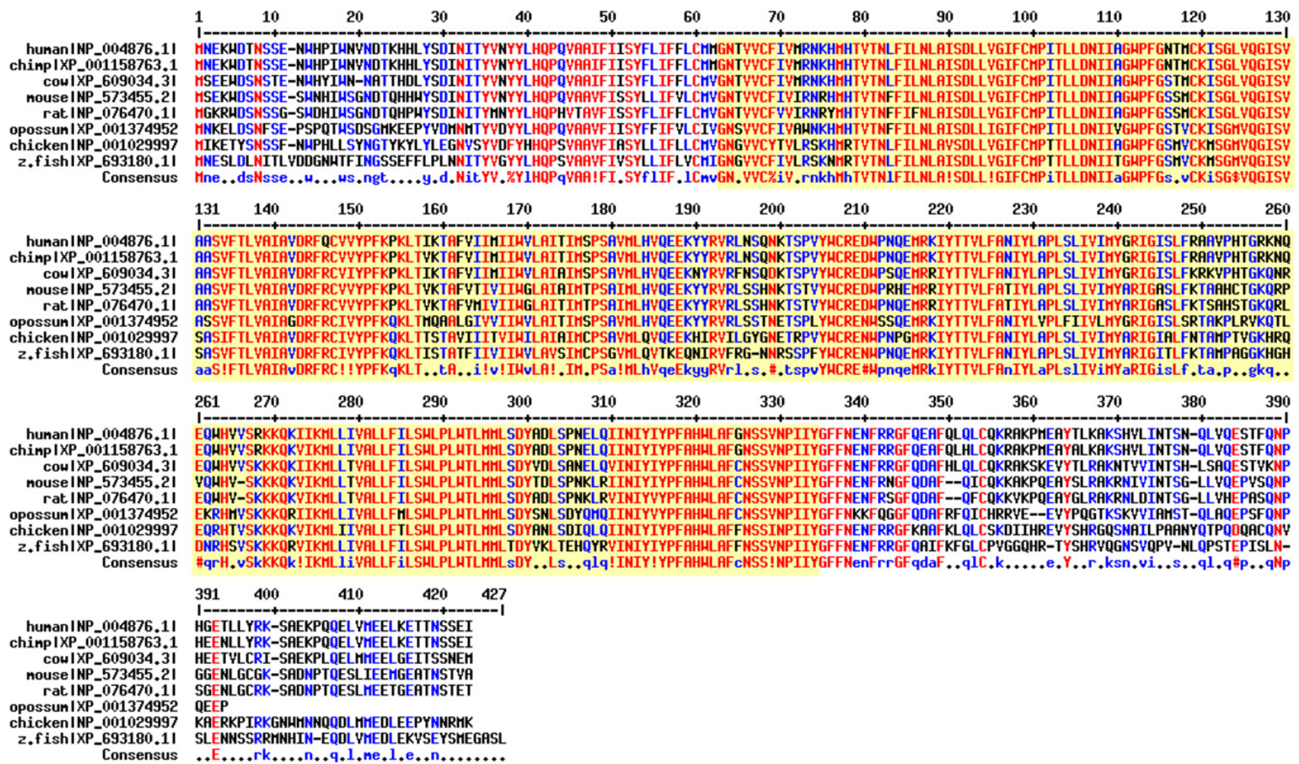

Fig. 3. Annotated and predicted receptors 2 for FF-amide are aligned using Multalin with default settings. The headers contain the organism name (shortened if necessary) followed by sequence ID. We did not find likely homolog from monkey and dog. The regions mapped to conserved domain, pfam00001.13, are highlighted in yellow. For clarity, the extra long N′-terminal region for human and chimpanzee were not used. Similar to that for receptor 1, this does not affect the overall alignment quality (data not shown).

the exact enzyme(s) involved still remains unclear. SLAAPQRF-NH_2 (NPSF, Fig. 4) was detected in tissues of rat, mouse and human and was found to have potent bioactivity (Bonnard et al., 2001; Deval et al., 2003; Jhamandas et al., 2006). Again, the processing of proNPFF to this octapeptide is unclear. Since NPSF is contained in the sequence of NPAF, rat EFWSLAAPQRF-NH_2 and mouse QFWSLAAPQRF-NH_2 (Fig. 4); these three unexplored peptides may provide additional, interesting tools to explore functional roles of the NPFF system. In fact, QFWSLAAPQRF-NH_2 has been convincingly detected in mouse spinal cord extracts by HPLC coupled with mass spectrometry (Bonnard et al., 2003).

Since the isolation of NPFF, numerous peptides with RF-NH_2 structure at their C-termini have been identified in mammalian tissues (Table 1). Other RF-amide peptides identified so far include prolactin-releasing peptides (PrRP) (Hinuma et al., 1998), RF-NH_2-related peptide (RFRP) (Hinuma et al., 2000) also referred to as NPVF derived peptides (Liu et al., 2001), metastin also termed kisspeptin (Kotani et al., 2001a; Ohtaki et al., 2001), and P518 also referred to as 26RFa (Chartrel et al., 2003). The identification of NPVF/RFRP gene derived peptides (Fig. 5) and two NPFF receptors, NPFF-R1 and NPFF-R2, raise the possibility that NPFF-related peptides may show cross reactivities to receptors of other RF-NH_2 peptides. This possibility undoubtedly can complicate the interpretation of early studies on the biological activities of NPFF. Two peptides derived from the human NPVF/RFRP precursor, VPNLPQRF-NH_2 (NPVF/$RFRP_3$) and SLNFEELKDWGPKNVIKMSTPAVNKMPHSFANLPLRF-NH_2 (NPSF/$RFRP_1$[1-37]) (Table 1 and Fig. 5), were identified by two separate groups of investigators (Hinuma et al., 2000; Liu et al., 2001). For purposes of clarity, since SLAAPQRF-NH_2 which is derived from the NPFF precursor (Fig. 4) is also referred to as NPSF, the peptide derived from the NPVF/RPRF gene, SLNFEELKDWGPKNVIKMSTPAVNKMPHSFANLPLRF-NH_2, will be referred to as $RFRP_1$(1-37) in this review. The NPVF/$RFRP_3$ peptide is structurally similar to NPFF (Table 1) and, furthermore, the G-protein coupled receptor (OT7TO22) originally identified as the receptor for NPVF/$RFRP_3$ also shows high affinity for NPFF. In fact, it has been suggested that the modulating activity of ICV injected NPFF on the analgesic potency of morphine may be due to binding of NPFF to NPFF-R1, the suggested receptor for NPVF/RFRP derived peptides (Liu et al., 2001). To minimize the confusion arising from the different names designated by different investigators for the two peptides,

(A) NPFF precursors

```
Human    MDSRQAAALLVLLLLIDG-GCAEGPGGQQE-DQLSAEEDSEPLPP   43
Bovine   MDARQAAALLLVLLLVTDWSHAEGPGGRDGGDQIFMEEDSGAHPA   45
Rat      MDSK-WAAVLLLLLLLRNWGHAEEAGSWGE-DQVFAEEDKGPHPS   43
Mouse    MDSK-WAAVLLLLLLLRNWGHAEEAGSWGE-DQVFAEEDKGPHPP   43

Human    QDA------QTSGSLLHYLLQAMERPGRSQAFLFQPQRFGRNTQG   82
Bovine   QDA------QTPRSLLRSLLQAMQRPGRSPAFLFQPQRFGRNTRG   84
Rat      QYAHTPDRIQTPGSLMRVLLQAMERPRRNPAFLFQPQRFGRNAWG   88
Mouse    QYAHTPDRIQTPGSLFRVLLQAMETPRRSPAFLFQPQRFGRSAWG   88

Human    SWRNEWLSPRAGEGLNSQFWSLAAPQRFGKK                 113
Bovine   SWSNKRLSPRAGEGLSSPFWSLAAPQRFGKK                 115
Rat      PWSKEQLSPQARE-----FWSLAAPQRFGKK                 114
Mouse    SWSKEQLNPQARQ-----FWSLAAPQRFGKK                 114
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(B) Endogenous Peptides

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NPFF(FLFQPQRF-NH2 ——— )
rNPA-NPFF(NPAFLFQPQRF-NH2 ▬▬▬ )
mSPA-NPFF(SPAFLFQPQRF-NH2 ▬▬▬ )
hNPAF(AGEGLNSQFWSLAAPQRF-NH2 •••••• )
bNPAF(AGEGLSSPFWSLAAPQRF-NH2 •••••• )
NPSF(SLAAPQRF-NH2 ------)
mQFW-NPSF(QFWSLAAPQRF-NH2 ▬ ▪ ▪)
```

Fig. 4. NPFF precursors in human, bovine, rat and mouse (A). The peptides that have been identified in tissue extracts are listed (B) and their locations in the precursors are indicated by various bars above the amino acid sequences. m:mouse, r:rat, h:human, b:bovine (Yang and Iadarola, 2006).

Table 1
Other RF-NH_2 peptides in mammalian system

RF-NH_2 peptide (speices)	Structure
Prolactin releasing peptide	
$PrRP_{20}$ (human)	TPDINPAWYASRGIRPVGRF-NH_2
$PrRP_{31}$ (human)	SRTHRHSMEIRTPDINPAWYASRGIRPVGRF-NH_2
Metastin or Kisseptin (human)	GTSLSPPPESSGSPQQPGLSAPHSRQIPAPQGAVLVQREKDLPNYNWNSFGLRF-NH_2
NPVF derived peptides/RFRP	
NPSF/$RFRP_1$(1-37) (human)	SLNFEELKDWGPKNVIKMSTPAVNKMPHSFANLPLRF-NH_2
NPVF/$RFRP_3$ (human)	VPNLPQRF-NH_2
P518 or 26RFa (mouse)	TSGPLGNLAEELNGYSRKKGGFSFRF-NH2

VPNLPQRF-NH_2 and SLNFEELKWGPKNVIKMS-TPA VNKMPHSFANLPLRF-NH_2, the protein precursor is shown in Fig. 5 and isolated or predicted peptides contained in the precursor are indicated (Fig. 5B) (Fukusumi et al., 2001; Hinuma et al., 2000; Liu et al., 2001; Yoshida et al., 2003).

(A) NPVF precursors

```
Human   MEIISSKLFILLTLATSSLLTSNIFCADELVMSNLHSKENYDKYSEPRGY  50

Bovine  MEIISLKRFILLMLATSSLLTSNIFCTDESRMPNLYSKKNYDKYSEPRGD  50

Rat     MEIISSKRFILLTLATSSFLTSNTLCSDELMMPHFHSKEGYGKYYQLRGI  50

Mouse   MEIISLKRFILLTVATSSFLTSNTFCTDEFMMPHFHSKEGDGKYSQLRGI  50

                                             (1)
                                             (2)
Human   PKG--ERSLNFEELKDWGPKNVIKMSTPAVNKMPHSFANLPLRFGRNVQE  98
                                             (3)
Bovine  LGWEKERSLTFEEVKDWAPK--IKMNKPVVNKMPPSAANLPLRFGRNMEE  98
                                             (4)
Rat     PKGVKERSVTFQELKDWGAKKDIKMSPAPANKVPHSAANLPLRFGRNIED 100

Mouse   PKGEKERSVSFQELKDWGAKNVIKMSPAPANKVPHSAANLPLRFGRTIDE 100

                                     (5)
Human   ERSAGATANLPLRSGRNMEVSLVRRVPNLPQRFGRTTTAKSVCRMLSDLC 148
                                     (6)
Bovine  ERSTRAMAHLPLRLGKNREDSLSRWVPNLPQRFGRTTTAKSITKTLSNLL 148
                                     (7)
Rat     RRSPRARA--------NMEAGTMSHFPSLPQRFGRTT-ARRITKTLAGLP 141

Mouse   KRSPAARV--------NMEAGTRSHFPSLPQRFGRTT-ARS-PKTPADLP 140

Human   QGSMHSPCANDLFYSMTCQHQEIQNPDQKQSRRLLFKKIDDAELKQEK   196

Bovine  QQSMHSPSTNGLLYSMACQPQEIQNPGQKNLRRRGFQKIDDAELKQEK   196

Rat     QKSLHSLASSELLYAMTRQHQEIQSPGQEQPRKRVFTETDDAERKQEKIG 191

Mouse   QKPLHSLGSSELLYVMICQHQEIQSPGGKRTRRGAFVETDDAERKPEK   188

Rat     NLQPVLQGAMKL                                       203
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(B) Identified and predicted peptides

(1) Human RFRP-1, predicted (Hinuma et al 2000)
(2) Human NPSF, predicted (Liu et al 2001)
(3) Bovine RFRP-1, identified (Fukusumi et al 2001)
(4) Rat RFRP-1, identified (Fukusumi et al 2001)
(5) Human NPVF or RFRP-3, predicted (Liu et al 2001; Hinuma et al 2000)
(6) Bovine RFRP-3, identified (Yoshida et al, 2003)
(7) Rat RFRP-3, identified (Yoshida et al, 2003)

Fig. 5. NPVF precursors in human, bovine, rat and mouse (A). The peptides that have been predicted from the precursor or identified in tissue extracts are listed (B) and their locations in the precursors are indicated by dashed bars above the amino acid sequences.

The relationships of NPFF precursor derived peptides and NPVF precursor derived peptides to NPFF-R1 and NPFF-R2 are summarized in Table 2. Using the cloned NPFF-R1 and NPFF-R2 and competitive binding assays, it has been shown that NPFF can bind to both receptors with high affinity. However, in general, NPFF-derived peptides (1st, 2nd, and 3rd peptides in Table 2) are found to have higher binding affinities for NPFF-R2. This high affinity binding of NPFF-derived peptides to NPFF-R2 is one of the reasons for suggesting that NPFF-R2 is the receptor for NPFF-derived peptides (Liu et al., 2001). On the other hand, NPFF-R1

Table 2
Binding affinities of NPFF-related and NPVF-related peptides of human origin and NPFF on human NPFF-R1 and NPFF-R2 expressed in CHO-NFA-bla cells coexpressing the chimeric G protein $G\alpha_{qi5}$

Peptide	Sequence	Binding affinity $IC_{50} \pm SD$ (nM)	
		NPFF-R1/OT7TO22	NPFF-R2/HLWAR77
hSQA-NPFF	SQAFLFQPQRF-NH_2	11.8 ± 3.3	1.1 ± 0.1
hNPAF	AGEGLSSPFWSLAAPQRF-NH_2	33.4 ± 9.3	3.3 ± 0.4
NPFF	FLFQPQRF-NH_2	6.2 ± 1.2	3.1 ± 0.3
NPSF/$RFRP_1$(1-37)	SLNFEELKDWGPKNVIKMSTPAV -NKMPHSFANLPLRF-NH_2	9.1 ± 1.3	7.5 ± 0.8
$RFRP_1$	MPHSFANLPLRF-NH_2	3.1 ± 0.6	6.1 ± 0.5
hNPVF/ $RFRP_3$	VPNLPQRF-NH_2	4.4 ± 0.5	122.3 ± 17.8
D-Tyr[1],(NMe)Phe[3]−NPFF	(d)YL(Nme)FQPQRF-NH_2	5.6 ± 1.0	3.4 ± 0.4
FMRFamide	FMRF-NH_2	3.1 ± 0.1	7.8 ± 1.5

^{125}I-YANLPLRF-NH_2 and ^{125}I-YLFQPQRF-NH_2 were used as radioligands for competition assays of NPFF-R1 and NPFF-R2 respectively. The data in the table are from Liu et al. (2001). The peptides derived from the human NPFF and NPVF?RFRP precursors are listed in the table because NPFF receptors studied in this table are of human origin.

was proposed to be the receptor for the RF-NH_2 peptides derived from the NPVF/RFRP gene (4th, 5th and 6th peptides in Table 2) (Liu et al., 2001). Surprisingly, FMRF-NH_2, (8th peptid in Table 2) which differs substantially from NPFF in its sequence, was found to have rather high affinity for both NPFF-R1 and NPFF-R2. This may be the reason that often similar pharmacological activities were found for FMRF-NH_2 and NPFF-related peptides in many studies.

For initial characterization of NPFF binding sites, radiolabeled NPFF or NPFF analogues were used, and high affinity and G-protein coupled binding sites were demonstrated in spinal cord membranes (Allard et al., 1989; Devillers et al., 1994; Payza and Yang, 1993). The binding studies all indicated the importance of the C-terminal RF-NH_2 for binding and the N-terminal segment for high binding affinity (Gicquel et al., 1994; Kotani et al., 2001b; Payza and Yang, 1993; Vyas et al., 2006). With the identification and characterization of the two NPFF receptor genes (Figs. 2 and 3) and the gene coding for NPVF/RFRP-derived peptides, it has become clear that the N-terminal segment of NPFF is important not only for binding affinity but also for receptor selectivity. However, the possible cross reactivity of NPFF-related peptides with NPFF-R1 undoubtedly can occur in pharmacological studies if NPFF-related peptides are injected without taking into consideration of their anatomical distribution. This, in turn, may render the interpretation of results of bioactivity studies difficult.

Many investigations have observed that NPFF-related peptides can modulate the analgesic potency of morphine (Roumy and Zajac, 1998). In general, *supraspinal* administration of NPFF or NPFF-related peptides was found to attenuate the analgesic effect of morphine. This effect was especially pronounced when a metabolically stable analogue, D-Tyr-Leu-[Nme]Phe-Gln-Pro-Gln-Arg-Phe-NH_2 (D-Tyr[1],[NMe]Phe[3]-NPFF), was injected ICV in the mouse (Gelot et al., 1998; Gicquel et al., 1994). Whether the morphine attenuating activity of NPFF-related peptides is mediated by NPFF-R1 or NPFF-R2 is unclear. The NPVF/RPRF-derived peptide, $RPRF_1$(1-37) with a high affinity for NPFF-R1, was found to be more potent in attenuating morphine analgesia than NPFF when injected ICV (Liu et al., 2001). The morphine modulating activity of NPFF is not due to direct interaction of NPFF with opiate receptors since opiate receptor binding assays are not affected by NPFF, and similarly NPFF binding sites are not influenced by morphine. Though mechanisms underlying the opiate modulating effect of NPFF still remain to be determined, there have been numerous studies addressing this question with a variety of *in vitro* approaches. At the cellular level, using neurons isolated from dorsal root ganglia of young rats, the inhibitory effect mediated by μ-opioid receptor on Ca^{2+} channels was attenuated by D-Tyr[1],(NMe)Phe[3]-NPFF (Rebeyrolles et al., 1996). Similarly, in neurons acutely dissociated from rat dorsal raphe nucleus, D-Tyr[1],(NMe)Phe[3]-NPFF was found to attenuate the μ-opioid receptor mediated inhibitory effect on Ca^{2+} channels (Roumy and Zajac, 1999). Since the identification of NPFF-R1 and NPFF-R2, neuroblastoma cells transfected with either of the two cloned NPFF receptor genes were utilized to explore mechanisms underlying the opioid attenuating activity of NPFF (Kersante et al., 2006; Mollereau et al., 2005a). The SH-SY5Y neuroblastoma cell line, expressing μ- and δ-receptors, was transfected with human NPFF-R2, and the recombinant cell line SH_2-D9 was used to explore molecular mechanisms responsible for the opioid attenuating effect of NPFF-related peptides (Roumy and Zajac, 1999). Treatment with the NPFF analogue, D-Tyr[1],(N-Me)Phe[3]-NPFF, attenuated the inhibition of N-type Ca^{2+} channels mediated by the μ-opioid receptor in SH_2-D9 cells but not in parental SH-SY5Y cells suggesting a specific role of NPFF-R2 in mediating the opioid attenuating effect. The same effect was also observed for SQA-NPFF, a peptide predicted from the human NPFF precursor. However, it should be noted that in

SH_2-D9 cells, the inhibition of N-type Ca^{2+} channels mediated by NPY Y2 receptors or α_2-adrenergic receptors was also attenuated by D-Tyr[1],(NMe)Phe[3]-NPFF. Using SH-SY5Y neuroblastoma cell transfected with NPFF-R1 (SH2-C7), a similar opioid attenuating activity was observed for NPVF/$RFRP_3$. Very recently, using SH-SY5Y cells stably transfected with CFP-tagged human NPFF-R2 and YFP-tagged μ-opioid receptor, the possible interaction between NPFF and μ-opioid receptors was explored (Roumy et al., 2007). Fluorescence resonance energy transfer and coimmunoprecipitation studies in these recombinant SH-SY5Y cells seem to provide evidence for a physical interaction between NPFF-R2 and μ-opioid receptors and this association is promoted by D-Tyr[1],(NMe)Phe[3]-NPFF and disrupted by the opioid agonist DAMGO ([D-Ala[2],N-Me-Phe[4],Gly[5]-ol]-enkephalin). It has also revealed that D-Tyr[1],(NMe)Phe[3]-NPFF can modify the lateral diffusion and consequently the disruption of the domain organization of μ-opioid receptors resulting in reduction of opioid response. These direct NPFF receptor-opioid receptor interactions appear to be at variance with earlier studies using ligand binding assays, but further work needs to be done to fully understand the functional ramifications. These reports are just a sampling of interesting initial studies aimed at exploring cellular or molecular mechanisms responsible for the opioid attenuating activity of exogenous NPFF-related peptides. However, possible opioid attenuating efffects of endogenous NPFF requires further investigation.

In addition to an opioids attenuating effect, NPFF or its related peptides have been found to show antinociceptive activity and potentiate opiate-induced analgesia. In general, this pronociceptive activity is observed when low doses of NPFF-related peptides are administered intrathecally. NPFF is distributed in confined regions of the CNS with the highest levels occurring in the dorsal spinal cord. High levels are also measured in the posterior pituitary. This distribution pattern strongly suggests that dorsal spinal cord is an appropriate site to study the bioactivity of NPFF in relationship to nociceptive sensory process. In contrast to ICV injection, intrathecally applied NPFF induces a long lasting naloxone reversible analgesia in both thermal and mechanical tests and, furthermore, NPFF at subeffective doses can potentiate morphine analgesia (Gouarderes et al., 1993). Following this very first report on the antinociceptive activity of NPFF, some additional studies have confirmed this observation in normal rats and in rats with inflammatory or neuropathic pain, for both NPFF or its analogues (Altier et al., 2000; Courteix et al., 1999; Gouarderes et al., 1996a; Gouarderes et al., 1993). A qualitatively similar but more potent effect was observed for the NPFF analogue D-Tyr[1],(NMe)Phe[3]-NPFF. These effects are reduced by μ- or δ-opioid receptor antagonists (Gouarderes et al., 1996a; Xu et al., 1999). In other studies (Altier et al., 2000; Courteix et al., 1999), intrathecal injections of NPFF were found to have no effect on acute thermal or mechanical pain in normal rats; whereas in rats with inflammatory or neuropathic pain, a potent antiallodynic effect was observed. It seems that the antinociceptive effect of NPFF or its analogue, D-Tyr[1],(NMe)Phe[3−]NPFF, is more easily demonstrated in the rats with inflammatory or neuropathic pain. The partial reversal of the analgesic effect of NPFF (or NPFF analogues) by an opioid antagonist is not consistently observed in different studies. The analgesic activity of intrathecal NPFF related peptides is not due to a sustained direct effect of NPFF because it is long lasting, on the order of hours (which is far longer than the half-life of exogenously administered NPFF peptides) and can be decreased by opioid receptor antagonists, at least partially. Several mechanisms underlying the analgesic effect of NPFF-related peptides have been suggested. At the spinal level, intrathecal infusion of D-Tyr[1],(NMe)Phe[3]-NPFF induced a long lasting outflow of met-enkephalin immunoreactive material in the rat (Ballet et al., 1999), thus accounting for its analgesic activity being sensitive to treatment with an opioid antagonist. Using dissociated mouse dorsal root ganglion neurons, neuropeptide FF and its analogues, D-YL(NMe)FQPQRF-NH_2, D-YD-L(NMe)FQPQRF-NH_2 and D-YD-LD-FQPQRF-NH_2, were found to reduce the rise in $[Ca^{2+}]$i induced by depolarization (Roumy and Zajac, 1996). In view of the presence of NPFF receptor at primary afferent endings (discussed more later), it was proposed that such an effect may be partially responsible for the analgesia induced by intrathecal injection of NPFF-related peptides. Recently, using a Chinese hamster ovary cell line stably expressing a c-MYC-tagged δ-opioid receptor, a direct interaction of NPFF with the δ-opioid receptor was explored (Anko and Panula, 2005). In these cells, the δ-opioid receptor agonist induced internalization of δ-opioid receptors, inhibition of forskolin stimulated cAMP accumulation, and phosphorylation of ERK2 (extra cellular signal-regulated kinase) and these effects were moderately but consistently enhanced by the co-application of the NPFF analogue, D-Tyr[1],(NMe)Phe[3]-NPFF. This effect was suggested to be a direct action of NPFF on the δ-opioid receptor, because, in the Chinese hamster ovary cell line used, the NPFF receptor was not detectable and NPFF has no intrinsic activity. This direct effect of NPFF on δ-opioid receptors was proposed to mediate, at least in part, the opioid potentiating effect of intrathecally injected NPFF-related peptides. However the physiological significance of this suggestion in *in vivo* conditions remains to be assessed.

The bioactivity of peptides from the NPAF region of the precursor have been rarely examined. A recent study (Jhamandas et al., 2006) has found that low doses (0.06 nmol) of NPAF, NPSF, and EFW-NPSF (see

Fig. 4) can markedly potentiate the analgesic activity of morphine but exert very weak intrinsic activity when injected intrathecally by themselves. EFW-NPSF is a predicted peptide from the rat NPFF precursor protein and both NPAF and EFW-NPSF are N-terminal extended NPSF peptides (Fig. 4) indicating the importance of the core NPSF sequence for the morphine potentiating effect. Interestingly, the analgesic activity of morphine in tolerant rats was also potentiated by the co-administration of NPSF related peptides with morphine. Whether NPFF is having a separate effect on the tolerance mechanism versus (or in addition to) increasing the agonist analgesic actions of morphine remains to be distinguished. Moreover, the mechanism responsible for the potent opioid potentiating effect requires further investigation.

3.2. *Effect on ASIC channels*

Another possible action of NPFF-related peptides in pain processing is at the ASICs (Lingueglia et al., 2006). Although, it has been generally assumed that NPFF-related peptides exert their actions via G-protein coupled receptors (NPFF-R2 and NPFF-R1), a second set of targets relevant to potential *pro*-nociceptive actions are the ASICs. FMRFamide-activated Na^+ channel (FaNaC) is expressed in invertebrates but not in mammalian systems; when FaNaC is expressed in Xenopus oocytes or mammalian cells, FMRF-NH_2 can activate it to produce a fast and partially desensitizing Na^+ current. The close relationship of FaNaC to ASICs in terms of their structure probably led to the various studies on the action of RF-amide peptides including NPFF-related peptides on ASICs. In mammalian systems, there are four different genes coding for 7 ASIC subunits, ASIC1a, ASIC1b, ASIC1b2, ASIC2a, ASIC2b, ASIC3 and ASIC4 (Lingueglia et al., 2006). ASIC1b and ASIC3, which are expressed in subsets of sensory neurons (Voilley et al., 2001; Waldmann and Lazdunski, 1998), have been proposed to have a role in pain perception (Chen et al., 2002; Chen et al., 2006). This proposal is further supported by ASIC3 knockout experiments (Price et al., 2001) and regulation of ASIC channel expression by inflammation (Mamet et al., 2002; Voilley et al., 2001). In the ASIC3 knockout experiment, reduced responsiveness to noxious and innocuous mechanical stimuli and to noxious heat and acidic conditions were observed (Price et al., 2001). In the Freund's adjuvant-induced inflammation model, ASIC1a, ASIC2b and ASIC3 gene expression was up-regulated (Voilley et al., 2001) and this up-regulation was found to be mediated by the proinflammatory mediators NGF, serotonin, interleukin-1 and bradykinin (Mamet et al., 2002). Various studies using cultured sensory neurons or ASIC cDNA transfected cells (Lingueglia et al., 2006) have shown that RF-NH_2 peptides (peptides with RF-NH_2 at their C-terminus) delay the rate of ASIC desensitization and also can increase the peak current amplitude in some cases following application of a low pH stimulus, thereby potentiating acid gated currents. In general, ASIC3 seems to play the major role for the potentiating effect of RF-NH2 peptides on acid gated currents (Xie et al., 2003). The RF-NH_2 peptides found to potentiate the acid gated currents include the tetrapeptides FMRF-NH_2 and FRRF-NH_2 (Askwith et al., 2000; Catarsi et al., 2001; Chen et al., 2006; Xie et al., 2003), NPFF (Askwith et al., 2000; Catarsi et al., 2001; Chen et al., 2006; Deval et al., 2003), NPSF (Deval et al., 2003) and 2 mouse peptides, VPHSAANLPLRF-NH_2 and SHFPSLPQRF-NH_2 (Xie et al., 2003) which are derived from the NPVF precursor protein. The potentiating effect of NPFF on the response of Xenopus oocytes expressing heteromeric human ASIC2a + ASIC3 to application of low pH was not observed for other known mammalian neuropeptides linked to nociception. The peptides tested were angiotensin, bradykinin, CCK, CGRP, dynorphin, galanin, neurokinin A, neurotensin, nociceptin, PACAP, substance P, VIP and NPY. Several of these are C-terminally amidated, NPY ends in a RY-NH_2 even, thereby, emphasizing the importance of the C-terminal RF-NH_2 and the specificity of the RF-NH_2 peptides for ASIC potentiating activity (Catarsi et al., 2001). However, RF-NH_2 peptides exert their ASIC potentiating activities at relatively high concentrations (micromolar), while this does not negate the interaction as a pharmacological target, it does raise a question regarding the physiological relevance of the modulatory role of RF-NH_2 peptides on ASIC. Regions of the peptide sequence other than the C-terminal RF-NH_2 can obviously influence the affinities of the RF-NH_2 peptides to ASICs because different peptides clearly show different potencies: in general FMRF-NH_2 exerts a higher potentiating activity than NPFF or NPVF-related peptides. During the analysis of DRG extracts by HPLC coupled with RIA, we have observed the presence of numerous immunoreactive peptides, which are detected by antisera against either NPFF or FMRF-NH_2 (unpublished observation). Whether RF-NH_2 peptides with a high affinity toward ASIC exist in sensory ganglia remains to be explored.

4. Distribution of NPFF and NPFF receptors

In early studies, the distribution of NPFF immunoreactivity was examined by RIA and immunohistochemical techniques using antibodies raised to NPFF. Since these studies, many RF-NH2 peptides including NPVF/$RFRP_3$ which shares an identical C-terminal PQRF-NH_2 sequence with NPFF have been identified (Table 1). Since antibody specificity does not require the entire sequence of the target peptide, the antibody

with specificity against the C-terminus of NPFF undoubtedly will detect other structurally similar peptides. Because of this, the distribution of NPFF immunoreactivity as demonstrated by immunohistochemistry or RIA alone should be interpreted with caution by taking into consideration other structurally similar peptides and the sequence determinants of the antibody specificity. Unlike other neuropeptides, NPFF is localized in limited areas in the CNS with the highest levels in the dorsal spinal cord and the posterior lobe of the pituitary gland in the rat. A detailed distribution of NPFF immunoreactivity including NPFF positive neuronal pathways was extensively delineated (Aarnisalo and Panula, 1995; Kivipelto et al., 1989; Kivipelto and Panula, 1991a; Lee et al., 1993; Majane et al., 1993; Panula et al., 1996). In the rat, the two major NPFF immunopositive cell groups in the brain were the medial hypothalamus between the dorsalmedial and ventromedial nuclei and the nucleus of the solitary tract. (Lee et al., 1993; Panula et al., 1996). However, the identification of NPVF/$RFRP_3$ has now revealed that the NPFF immunoreactivity in cell groups in these regions of the hypothalamus is due to NPVF-related peptides, which are structurally similar to NPFF but are derived from a different precursor (Figs. 4 and 5). In the rat spinal cord, NPFF immunoreactivity was localized by RIA (Majane et al., 1989) to the dorsal spinal cord where NPFF immunoreactive terminals and fibers were detected immunohistochemically mainly in the superficial laminae (Kivipelto and Panula, 1991b; Panula et al., 1996), the layer in which nociceptive primary afferent fibers terminate. In the rat spinal cord, immunoreactivity was also observed in other regions including central canal, dorsal lateral funiculi, intermediolateral columns and ventral horn. This network of NPFF terminals and fibers was found to be mostly of intrinsic spinal origin; NPFF positive cell bodies were detected in the substantia gelatinosa, marginal zone, laminae III, IV and X, dorsal lateral funiculus, and dorsal gray commissure of the lumbosacral transition zone (Kivipelto and Panula, 1991b).

The gene coding for the NPFF precursor was cloned (Perry et al., 1997; Vilim et al., 1999) and the distribution of NPFF mRNA was found to be very similar to that of NPFF immunoreactivity except in the hypothalamic region. In the rat, the highest level of NPFF mRNA was found in the dorsal spinal cord and the nucleus of the solitary tract (Vilim et al., 1999). The NPFF immunoreactivity in the rat hypothalamus is now known to be due to cross reactivity of the C-terminally directed NPFF antiserum to the NPVF/RFRP gene derived $RF\text{-}NH_2$ peptides because NPFF mRNA was not present but NPVF mRNA was abundantly expressed in the rat hypothalamus (Vilim et al., 1999). In human CNS, using northern blot analysis, NPFF mRNA was detected in the medulla and spinal cord. (Nystedt et al., 2002). The identification of the two genes coding for two structurally similar peptides, NPFF and NPVF, the distribution and, in turn, the functional roles of these two peptides can now be better studied and differentiated.

Using $[^{125}I]YLFQPQRF\text{-}NH_2$, a single NPFF specific and high affinity binding site was first demonstrated in rat spinal cord membranes (Allard et al., 1989) and the distribution of this binding site in CNS was studied in the rat (Allard et al., 1992) and also in human spinal cord (Allard et al., 1994a). In the rat CNS, $[^{125}I]YLFQPQRF\text{-}NH_2$ binding sites were detected in many regions with relatively higher levels in the superficial layers of the dorsal horn at all spinal levels and undetectable levels in any area of the cerebellum (Allard et al., 1992). In the human spinal cord and lower medulla oblongata, $[^{125}I]YLFQPQRF\text{-}NH_2$ binding sites are distributed unevenly, with the highest densities in the superficial layers of the dorsal horn and spinal trigeminal nucleus (Allard et al., 1994a). In the rat spinal cord, using the metabolically stable analogue $[^{125}I][\text{D-}Tyr^1,(Nme)Phe^3]$NPFF, the highest levels of NPFF binding sites were observed in laminae I-II, moderate to low levels were seen in the laminae III-IV, around the central canal and ventral horn. The $[^{125}I]YLFQPQRF\text{-}NH_2$ binding sites were suggested to be mainly located postsynaptically on second order spinal cord neurons and not on the primary afferent terminals because the densities of binding in the dorsal spinal cord were not decreased by neonatal capsacin treatment or dorsal rhizotomy (Allard et al., 1994b; Lombard et al., 1995). Contradictory results were observed in other studies and will be addressed along with NPFF immunoreactivities in more detail later. In the rat spinal cord, the distribution of NPFF positive nerve terminals and fibers seems to match that of NPFF binding sites.

In the year 2000, two G-protein coupled receptors with high affinities for NPFF were identified (Bonini et al., 2000; Elshourbagy et al., 2000; Hinuma et al., 2000). These two receptors are encoded by separate genes and share 59% similarity in their amino acid sequences (Figs. 2 and 3). Although both receptors, NPFF-R1 and NPFF-R2, bind NPFF with high affinity, there are some differences in their binding affinities for NPFF- and NPVF-related peptides (Bonini et al., 2000). The *in vitro* study using cells expressing NPFF-R2 has revealed that NPFF-R2 is responsive to and also more selectively binds NPFF-related peptides in comparison with NPVF-related peptides (Table 2). While cells expressing NPFF-R1 were found to show a small degree of preference for NPVF-related peptides, NPFF-R1 seems to exhibit less selectivity (Bonini et al., 2000). Regional distributions of NPFF-R2 and NPFF-R1 mRNAs in rat and human tissues were analyzed by *in situ* hybridization (Liu et al., 2001) and

RT-PCR (Bonini et al., 2000; Yang and Iadarola, 2003) and found to be different from each other. In the rat CNS, the highest level of NPFF-R2 mRNA was detected in the spinal cord with a striking localization in the superficial layers of the dorsal horn. A relatively high level of NPFF-R2 mRNA was also detected in the hypothalamus. This distribution of NPFF-R2 seems to generally parallel that of NPFF except in the region of hypothalamus (Yang and Iadarola, 2003; Yang and Iadarola, 2006) where NPFF-R2 mRNA is abundantly expressed, but NPFF is hardly identified in rat hypothalamic extracts (Majane et al., 1989). This raises the question of axonal transport of the peptide and the receptor or the possibility of combinatorial association of either of the two receptors with peptides derived from either of the two precursors. The highly localized distribution of NPFF and NPFF-R2 in the superficial layers of spinal cord suggested a role for the spinal NPFF system in nociception and NPFF-R2 as a physiologically relevant receptor for NPFF. However, it should be noted that a different distribution pattern of NPFF receptors was found in the human CNS: in the spinal cord, high levels of NPFF-R1 mRNA were detected, while NPFF-R2 transcripts were found to be detectable but at a very low level in normal human tissue. Nonetheless, the possible role of NPFF-R2 at the spinal level in humans remains relevant because we have found high levels of NPFF-R2 mRNA in human post mortem DRG. Furthermore, in the rat, we have observed the presence of NPFF-R2 immunoreactivity in sensory afferent terminals (Iadarola et al., 2003) following peripheral inflammation. These observations (unpublished results) suggest that human dorsal spinal cord may have a significant level of functional NPFF-R2 protein originating from DRG. This suggestion is consistent with studies in African green monkey CNS (Zeng et al., 2003), immunohistochmical experiments with anti human NPFF-R2 have found that NPFF-R2 immunoreactivity is present in lower brain stem and gray matter of spinal cord with dense labeling in the spinal trigeminal nucleus of the lower brain stem and superficial layers of the dorsal horn. A similar distribution pattern of NPFF-R2 mRNA was revealed by *in situ* hybridization. The results again suggest a role for the NPFF system including NPFF-R2 in the modulation of nociceptive process at spinal level in primates.

Early studies seem to suggest the absence of NPFF in rat sensory ganglia because NPFF was not detected in normal spinal ganglia (Panula et al., 1987) immunohistochemically and dorsal spinal cord rhizotomy failed to reduce NPFF levels in dorsal spinal cord (Majane et al., 1989). The failure to detect NPFF mRNA in dorsal root ganglia by *in situ* hybridization (Vilim et al., 1999) further suggested the absence of NPFF in the normal sensory ganglia. These results are undoubtedly due to the very low levels of NPFF peptide in the DRGs of normal rats. The failure to observe a decrease of the NPFF content in dorsal spinal cord after rhizotomy is, in part, due to the very high level of NPFF peptide in the dorsal spinal cord and the fact that afferent nerve fibers innervate multiple spinal segments (Traub et al., 1989). Now, there are several lines of evidence that suggest the presence of NPFF and NPFF receptors in the sensory ganglia. Using a highly sensitive RIA, a very low level of NPFF immunoreactivity was detected in DRG extract from colchicine treated rats (Allard et al., 1999). Analysis of this NPFF immunoreactivity with HPLC followed with RIA detected 3-4 NPFF immunoreactive peptides and one of them was identified as NPFF from its elution profile. As previously reported, NPFF immunoreactivity was not detected immunohistochemically in the DRG of normal rats, however, in colchicine treated rats, NPFF immuno-positive neuronal cell bodies with small to medium diameters were detected in the DRG. In line with this study, we have detected NPFF mRNA in DRG of both rat and human by RT-PCR. The relatively low sensitivity of *in situ* hybridization in comparison with the very high sensitivity of RT-PCR undoubtedly can explain the failure to detect NPFF transcripts in the earlier study using *in situ* hybridization. Furthermore, we found that the NPFF mRNA level was upregulated by peripheral inflammation (Iadarola et al., 2003). This observation will be discussed further later.

In the rat CNS, the highest level of NPFF binding sites is found in the dorsal spinal cord and these NPFF binding sites are located not only on the spinal intrinsic neurons but also on the primary afferent terminals. Both dorsal spinal cord rhizotomy and neonatal capsaicin treatment were found to cause a significant depletion of NPFF binding sites in the dorsal horn (Gouarderes et al., 1996b). Using an antibody raised against NPFF-R2, we have observed immunohistochemically that some of the dorsal spinal cord NPFF-R2 is located on the primary afferent terminals in rats with hind paw carrageenan inflammation (Iadarola et al., 2003). Now there are studies suggesting that the NPFF-R2 synthesized in the DRG is transported to primary afferent terminals. NPFF immunoreactivity as well as NPFF mRNA is very low in the DRG of normal rat. In contrast, the NPFF R2 mRNA is relatively abundant in DRG and trigeminal ganglia (Bonini et al., 2000) though the highest levels are in the spinal cord. In the rat, ligation of lumbar dorsal spinal roots induced a significant accumulation of NPFF binding sites on the side peripheral to the ligature suggesting the axonal transport of NPFF binding sites, synthesized in DRG, towards primary afferent endings (Gouarderes et al., 2000). This migration of NPFF binding sites (receptor protein) was further supported by the finding of relatively dense NPFF binding sites in the spinal trigeminal tract (Allard et al., 1992; Dupouy and Zajac, 1996)

where NPFF receptor mRNA was not detected (Liu et al., 2001). The molecular mechanisms underlying the transport of NPFF receptors remains an interesting question to be explored.

The dual localization of NPFF receptor in the dorsal spinal cord, on both afferent terminals and second order neurons, raises an important question regarding the site of action of intrathecally injected NPFF-related peptides, which can induce a long lasting analgesia and opiate potentiating activity. From the foregoing discussions these effects can be influenced by a variety of factors, at the neural circuit level (anatomically) and pharmacologically because of the multiple receptors present. For example, it is possible that the analgesia and opiate effects may be mediated by dual activation of the same receptor in both cellular locations or the effects may be separable.

5. Regulation of NPFF system and its relationship to nociception; (A) *in vitro* and (B) *in vivo* studies

5.1. Regulation of NPFF in in vitro studies

Following the cloning of the NPFF gene, the transcriptional regulation of the human NPFF gene was studied at the molecular level (Nystedt et al., 2002). In this study, 4.7 kb of promoter regions was cloned and analyzed. The result has revealed that there are two distinct transcription initiation sites and multiple potential transcription factor binding sites. The highest promoter activity was localized between −552 bp to −830 bp of the 5’-flanking region and a potential silencer element was found in a region from −220 bp and −551 bp. Most interestingly, in PC12 cells, NPFF gene expression was up-regulated by NGF and this effect was localized to the region between −61 bp and −214 bp of the 5’-flanking region where potential AP-2, NFκB and STAT-1 binding sites were predicted. Analysis of the 5’-flanking region of the mouse NPFF gene again identified an NGF responsive region and this effect was localized to nucleotides −83 to +10 surrounding the transcriptional start site (Nystedt et al., 2006). These studies may provide useful leads for the further exploration of the role of NPFF in pain mechanisms at the molecular level.

Very recently, regulation of endogenous human NPFF-R2 by NPFF was studied at the cellular level in a neuroblastoma cell line (SK-N-MC) endogenously expressing NPFF-R2, the NPFF precursor and components of the intracellular signaling network (Anko and Panula, 2006). Exposure of SK-N-MC cells to the NPFF analogue D-Tyr^1,(NMe)Phe^3-NPFF was found to inhibit forskolin activated adenylate cyclase, phosphorylation of extracellular signal-regulated kinase (ERK2), formation of actin stress fibers, and up-regulation of NPFF-R2 gene expression. It was suggested that ERK1/2 may be involved in the up-regulation of NPFF-R2 gene expression following treatment of the cell with NPFF.

5.2. Regulation of NPFF system in in vivo studies

Several lines of evidence have now suggested that the spinal NPFF system is altered in animals during persistent nociceptive activation. In the very first *in vivo* study, the effect of peripheral inflammation on spinal NPFF was studied immunohistochemically in rats injected with carrageenan at the hind paw (Kontinen et al., 1997). The inflammation revealed neuronal cell bodies in spinal cord that contained NPFF (even without colchicine treatment), while staining for NPFF immunoreactive fibers and terminal-like thickenings were not visibly altered. This up-regulation of NPFF immunoreactivity was not observed if rats were pretreated with morphine before carrageenan injection. The results suggest that NPFF biosynthesis is up-regulated at the spinal level by nociceptive component of peripheral inflammation. In agreement with this finding, the number of the NPFF mRNA positive cells was found to be increased in the rat spinal cord by peripheral carrageenan inflammation as revealed by *in situ* hybridization analysis (Vilim et al., 1999). In contrast to the inflammatory pain model, up-regulation of spinal cord NPFF mRNA was not detected in rats using the spinal nerve ligation neuropathic pain model of Kim and Chung (Nystedt et al., 2004; Vilim et al., 1999). How the difference in nociception-inducing mechanisms between the inflammatory and neuropathic pain models affects the regulation of the spinal NPFF system remains unclear at present time, however, similar considerations were raised when the various models were compared using dynorphin gene upregulation (Draisci et al., 1991). Furthermore, whether NPFF synthesis in DRG is affected by neuropathic pain remains to be assessed. In addition to NPFF peptide, NPFF binding sites are also affected by peripheral inflammation. In rats with joint inflammation induced by *Mycobacterium butyricum* suspended in Freund’s adjuvant injected into the tibio-tarsal joint, [^{125}I]-1-dimethyl-YLFQPQRF binding sites were found to be increased in the spinal cord (Lombard et al., 1999). This up-regulation of both ligand and its receptor was further observed in the subsequent studies on regulation of NPFF and NPFF-R2 gene expressions.

In studying the regulation of genes encoding NPFF and its receptor, NPFF-R2, in the same animal, we and others have found that expression of both NPFF peptide and NPFF-R2 are coordinately up-regulated by peripheral inflammatory stimuli. In rats injected with carrageenan at the hind paw, we observed up-regulation of both NPFF and NPFF-R2 gene expressions in the spinal cord (Yang and Iadarola, 2003). In another rat model of inflammatory pain induced by injection of a

low dose of carrageenan into the hind paw, a transient up-regulation of gene expression was observed for both NPFF and NPFF-R2 in the lumbar spinal cord and furthermore, the increased gene expression of NPFF seemed to precede that of NPFF-R2 (Nystedt et al., 2004). The physiological significance of coordinated up-regulation of NPFF and its receptor when induced by peripheral inflammation is not readily obvious at the present time and remains to be explored. The report on the potent morphine analgesia potentiating activity of intrathecal NPFF (Gouarderes et al., 1993; Jhamandas et al., 2006) taken together with the up-regulation of both NPFF and NPFF-R2 by inflammatory pain, leads us to speculate that the enhanced analgesic activity of morphine in inflammatory pain (Hylden et al., 1991; Przewlocki and Przewlocka, 2001) may involve the spinal NPFF system. The regulation of the NPFF system was also explored in other models of pain in the rat. In rats with neuropathic pain induced by tight spinal nerve ligation or acute colitis induced by instillation of 2,4,6-trinitrobenzene sulfonic acid into the colon, up-regulation of NPFF-R2 gene expression but not that of NPFF was observed in the brain stem; however, the effect was small (Nystedt et al., 2004).

In the mouse spinal cord, one of the peptides from the NPVF precursor, $RFRP_3$, exhibited a dense network of immunoreactive fibers in the superficial layer of spinal trigeminal nucleus and dorsal spinal cord (Ukena and Tsutsui, 2001). Subsequently, the rat equivalent of human NPVF/$RFRP_3$ was characterized to be an amidated octadecapeptide (ANMEAGTMSHFPSLPQRF-NH2) (Ukena et al., 2002) (Fig. 5). This peptide shares an identical C-terminal tetra-peptide-amide sequence with NPFF-related peptides. The C-terminal portion of NPFF related peptides has been strongly suggested to be important for their bioactivities. Thus, we have also examined the possible involvement of $RFRP_3$ in persistent nociception and found that, in rats, transcript levels for NPVF/RFRP precursor or NPFF-R1 (proposed receptor for $RFRP_3$) were not altered by peripheral carrageenan inflammation. The results seem to indicate that the possible pain modulatory role of NPFF-related peptides may not be readily shared by peptides derived from the NPVF/RFRP precursor.

As previously discussed, the NPFF system is expressed at very modest levels in the DRG of the normal rats, thus, the functional role of the NPFF system in DRG has been poorly explored. In one of our studies (Iadarola et al., 2003), we observed a coordinated up-regulation of NPFF and NPFF-R2 transcripts in the DRG of the rat with peripheral inflammation induced by injection of carrageenan into the hind paw. To understand the cellular location of the NPFF-R2 at the spinal level, anti-NPFF-R2 antiserum was prepared and immunohistochemical analysis of NPFF-R2 was performed. The NPFF-R2 immunoreactivity was localized to laminae I and II, V and X. Following hind paw inflammation, additional NPFF-R2 immunoreactivity was identified in primary afferent terminals. Current evidence suggests that the NPFF-R2 gene is expressed at a modest level in normal human spinal cord, raising a question of the relevance of studying NPFF-R2 as the receptor for NPFF and role of the NPFF system in nociception in humans. However, in the human spinal cord, the functional NPFF-R2 protein at the presynaptic site may be increased during persistent inflammatory pain states resulting from the up-regulation of NPFF-R2 gene expression in the DRG. It should be noted that this is a speculation based on our observation on the rat model of inflammatory pain. Our studies on the DRG suggest that the NPFF system in the spinal sensory ganglia may also be involved in pain processing.

The effect of opiate treatments on endogenous NPFF levels has been studied in the rat. Within 30 min after subcutaneous injection of heroin, a significant decrease of NPFF immunoreactivity (38%) was observed in the spinal cord and this effect was suggested to be due to the release of NPFF by the opiate (Devillers et al., 1995). In rats with implanted morphine pellets ($2 \times$ of 75 mg morphine), NPFF immunoreactivity in hypothalamus, brain stem and spinal cord was found to be decreased 1 h after the morphine pellet implantation followed by an increase of NPFF immunoreactivity between 3 and 6 h (Stinus et al., 1995). It was suggested that the initial decrease and subsequent increase of NPFF immunoreactivity was due to an increased release followed by an enhanced synthesis of NPFF immunoreactivity. It should be noted that the NPFF immunoreactivity measured may include other RF-NH2 peptides especially the other PQRF-NH_2 containing peptides namely the NPVF/RFRP gene derived peptides. At the mRNA level, a single injection of morphine exerted no effect on NPFF or NPFF-R2 in the spinal cord but induced a significant but small increase of NPFF and NPFF-R2 mRNAs in the brain stem (Nystedt et al., 2004). Very recently, a NPFF receptor antagonist, a derivative of RF-amide, was developed and termed RF9 (Simonin et al., 2006). Using recombinant NPFF-R1 and NPFF-R2, RF9 displays the same affinity and antagonist activity to both of these NPFF receptors. In an *in vivo* study, RF9, when applied together with heroin, was found to block the delayed and sustained hyperalgesia and the decreased analgesic effect of repeated heroin administration. Although it is unclear whether this effect is mediated by NPFF-R1 or NPFF-R2, the result is in line with the suggestion that NPFF-related peptides may have a modulatory role in opiate analgesia.

Regulation (summarized in Table 3) and localization of the NPFF system including NPFF and its receptor, taken together with the analgesic, pro-nociceptive and morphine modulating activities of NPFF-related pep-

Table 3
Summary on regulation of NPFF system in *in vivo* study

(A) Regulation of NPFF and NPFF receptor immunoreactivity Treatment	Effect on NPFF or NPFF-R2 immunoreactivity
Carrageenan (0.2 mg) injected into hind paw	Increase of NPFF immunoreactive cell bodies in spinal cord (Kontinen et al., 1997)
Mycobacterium butyricum in Freund's adjuvant injected into tibio-tarsal joint	Increase of [^{125}I]-1-dimethyl-YLFQPQRF-NH_2 binnding sites in spinal cord (Lombard et al., 1999)
Carrageenan (6 mg) injected into hind paw	Increase of NPFF-R2 immunoreactivity in primary afferent terminals (Yang and Iadarola, 2003)
Single subcutaneous injection of heroin (2.5 mg/kg)	Decrease of NPFF immunoreactivity in spinal cord at 30–60 min after treatment (Devillers et al., 1995)
Morphine pellet implantation (2 × 75 mg)	Decrease of NPFF immunoreactivity in spinal cord, brain stem and hypothalamus 1 h after morphine pellet implantation (Stinus et al., 1995)
Morphine pellet implantation (2 × 75 mg)	Increase of NPFF immunoreactivity in spinal cord, brain stem and hypothalamus 3 to 6 h after morphine pellet implantation (Stinus et al., 1995)

(B) Regulation of NPFF and NPFF receptor mRNAs Treatment	Effect on NPFF mRNA or NPFF-R2 mRNA
Carrageenan (0.2 mg) injected into hind paw	Increase of NPFF mRNA in spinal cord (Vilim et al., 1999)
Carrageenan (6 mg) injected into hind paw	Coordinated increase of both NPFF mRNA and NPFF-R2 mRNA in spinal cord (Yang and Iadarola, 2003)
Carrageenan (6 mg) injected into hind paw	No effect on both NPVF/RFRP mRNA and NPFF-R1 mRNA in spinal cord (Yang and Iadarola, 2003)
Carrageenan (0.2 mg) injected into hind paw	Coordinated increase of both NPFF mRNA and NPFF-R2 mRNA in spinal cord (Nystedt et al., 2004)
Carrageenan (6 mg) injected into hind paw	Coordinated increase of both NPFF mRNA and NPFF-R2 mRNA in DRG (Iadarola et al., 2003)
Tight ligation of spinal nerve	Increase of NPFF-R2 mRNA in brain stem but no effect on both NPFF mRNA and NPFF-R2 mRNA in spinal cord (Nystedt et al., 2004)
Colitis by instillation of 2,4,6-trinitrobenzene sulfonic acid (60 mg/kg) into colon	Increase of NPFF-R2 mRNA in brain stem but no effect on both NPFF mRNA and NPFF-R2 mRNA in spinal cord (Nystedt et al., 2004)
Single subcutaneous injection of morphine (2.5 mg/kg)	Small increase of both NPFF mRNA and NPFF-R2 mRNA in brain stem but not in spinal cord (Nystedt et al., 2004)
Repetitive injection of morphine (2 × 10 mg/kg) for 7days	No effect on both NPFF mRNA and NPFF-R2 mRNA in spinal cord or brain stem (Nystedt et al., 2004)

tides strongly suggest the involvement of the NPFF system in pain processing especially at the spinal level. As to exactly how the NPFF system participates in modulating pain still requires further clarification.

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