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Mini-review

Modulatory roles of the NPFF system in pain mechanisms at the spinal level

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ABSTRACT

The possible roles of the NPFF system in pain processing are summarized from the viewpoints of (1) biological activities of NPFF, (2) anatomical distribution of NPFF and its receptor(s) and (3) the regulation of NPFF and receptor(s) in animal models of pain. NPFF and NPFF analogues were found to have analgesic, pronociceptive and morphine modulating activities. Since the isolation of NPFF, several other RF-NH_2 peptides have been identified and some of them were found to have nociceptive or morphine modulating activity. Depending on the pharmacological doses and locations of administration, NPFF may exhibit the biological activities of other structurally related RF-NH_2 peptides thus complicating NPFF bioactivity studies and their interpretation. Acid sensing ion channels were found to respond to RF-NH_2 peptides including NPFF, raising the possibility that interaction of NPFF and acid sensing ion channels can modulate nociceptive activity. NPFF and NPFF receptor mRNAs are highly expressed and localized in the superficial layers of the dorsal cord, the two genes are also in dorsal root ganglia though at much lower level. The spinal NPFF system is up-regulated by peripheral inflammation in the rat. Furthermore, immunohistochemically, NPFF receptor 2-protein was demonstrated to be increased in the primary afferents in the spinal cord of rats with peripheral inflammation. Regulation and localization of spinal NPFF systems, taken together with the analgesic bioactivity of intrathecally administered NPFF, strongly suggest involvement of spinal NPFF system in pain processing.

1. Introduction

During early studies of the opioid peptide met-enkephalin-arg-phe (YGGFMRF), it was observed that, after intracerebroventricular (i.c.v.) injection, analgesic activity was found for YGGFMRF but not for YGGFMRF-NH_2. YGGFMRF-NH_2 can be viewed as a composite of the opioid peptides, YGGFMRF or YGGFM (met-enkephalin), and FMRF-NH_2, a cardioexcitatory peptide of molluscan origin [59]. In the perfused clam recta, YGGFMRF and FMRF-NH_2 exhibited opposite effects [24]. Using an antiserum against FMRF-NH_2 the presence of FMRF-NH_2-like peptide(s), distinct from FMRF-NH_2, was detected in mammalian CNS immunohistochemically [19]. In order to explore the biological role of FMRF-NH_2-like immunoreactive material, especially its possible modulatory role in pain processing and the opioid system, two FMRF-NH_2-like

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Fig. 1 – NPFF precursors in human, bovine, rat and mouse [67]. Arrows indicate the peptides NPFF, NPAF and NPSF and the two N-terminally extended NPFF peptides identified in rat and mouse, respectively (see Table 1).

peptides were isolated from extracts of bovine medulla oblongata, sequenced and biochemically characterized [73].

Since the isolation and characterization of NPFF (FLFQPQRF-NH_2) in 1985, the biological roles suggested for this peptide include pain modulation [23,54,55,62], water balance [33,49,64], food consumption [18,51,52], modulation of opiate mediated effects [52,62,73], cardiovascular actions [27,30,40] and body energy storage and utilization [42]. High affinity NPFF binding sites distinct from opioid receptors were demonstrated in rat spinal cord and brain [2]; furthermore, these receptors were demonstrated to couple to G-proteins [57].

The gene coding for the NPFF precursor protein was cloned by two separate groups in 1997 [58] and 1999 [67]. The NPFF precursor proteins of human, bovine, rat and mouse are shown in Fig. 1. The products generated from the NPFF precursor and actually biochemically identified in tissues of various species are listed in Table 1. The locations of these products in the precursors are indicated by arrows in Fig. 1. The N-terminally extended peptides, NPA-NPFF and SPA-NPFF, are the products generated from the cleavage of consensus processing sites [11] but processing of other peptides listed in Table 1 still remains unclear and to be determined. Interestingly, another novel gene coding for two N-terminal extended RF-NH_2 peptides, VPNLPQRF-NH_2 and SLNFEELKDWGPKNNVIKMSTPAVNKMPHSFANLPLRF-NH_2 was identified by genomic data base searches and cloned in mammals by two groups [26,44]. VPNLPQRF-NH_2, which is identical to NPFF in its C-terminal tetrapeptide sequence, was referred to as RFamide-related peptide 3 (RFRP-3) [26] or NPVF [44]. The peptide, SLNFEELKDWGPKNNVIKMSTPAVNKMPHSFANLPLRF-NH_2, was referred to as RFamide-related peptide 1 (RFRP-1) [26] or NPSF [44]. It should be noted that, in various studies, NPSF has been used to designate two separate peptides SLNFEELKDWGPKNNVIKMSTPAVNKMPHSFANLPLRF-NH_2 derived from the NPVF precursor and SLAAPQRF-NH_2 derived from NPFF precursor. In order to avoid confusion,

Table 1 – Peptides actually identified in tissue extracts of various species and contained in the NPFF precursor

Peptide	Sources
NPFF: FLFQPQRF-NH_2	Boviene spinal cord [48,73], rat spinal cord [5,10,35], mouse spinal cord [10], human CSF [63]
NPA-NPFF: NPAFLFQPQRF-NH_2	Rat spinal cord [10]
SPA-NPFF: SPAFLFQPQRF-NH_2	Mouse spinal cord [11]
NPAF: AGEGLSSPFWSLAAPQRF-NH_2	Bovine spinal cord [73]
NPAF: AGEGLNSQFWSLAAPQRF-NH_2	Human CSF [13]
NPSF: SLAAPQRF-NH_2	Rat spinal cord [10], mouse spinal cord [10], human CSF [13]

Locations of various peptides, listed in the table, in the NPFF precursor are indicated by arrows in Fig. 1.

SLNFEELKDWGPKNNVIKMSTPAVNKMPHSFANLPLRF-NH_2 and SLAAPQRF-NH_2 will be referred to as NPSF = RFRP-1 and NPSF, respectively in this review.

Following the cloning of the NPFF precursor gene, identification of NPFF receptor genes was reported by three different groups in the same year (year 2000).

The first NPFF receptor gene identified was a G-protein coupled receptor (GPCR) referred to as HLWAR77 [20], subsequently, two GPCRs were identified and referred to as NPFF receptor1 (NPFF-R1) and NPFF receptor 2 (NPFF-R2) [12]. Both of these receptors, encoded by two separate genes, were found to have high affinities for NPFF. Comparison of the amino acid sequences of these genes indicated that NPFF-R2 and NPFF-R1 are identical to the GPCRs, HLWAR77 and OT7TO22 [26], respectively. In searching for the receptor for hRFRP-3 (also referred to as NPVF [44]), OT7TO22 was identified and suggested to be its functional receptor [26]. Identification of the NPVF gene encoding for NPVF and NPSF = RFRP-1 enabled the further characterization of NPFF-R1 and NPFF-R2 with peptides derived from the NPFF precursor and those predicted from NPVF precursor. As shown in Table 2, NPFF-R2 exhibits a somewhat higher binding affinity for NPFF derived peptides, while NPFF-R1 is more responsive to peptides deduced from the NPVF precursor. Consequently, it has been suggested that the NPFF-related peptides and NPVF-related peptides are the preferred ligands for NPFF-R2 and NPFF-R1, respectively.

In addition to NPVF related peptides, several other RF-NH_2 peptides and their receptors have been identified, thus complicating the interpretation of the results of the early studies on the biological activities of NPFF. Identification of the NPFF precursor gene and its receptors and their regional expression patterns undoubtedly allows the functional roles of the NPFF system to be explored more precisely. Evidence is now accumulating to strongly support a role for the NPFF system in nociceptive mechanisms. In this review, the possible roles of the NPFF system in pain processing will be summarized from several viewpoints: the biological activities of NPFF determined in early studies, the anatomical distribution of NPFF and its receptor(s) and the regulation of NPFF and the NPFF receptor in animal models of pain with an emphasis on functional roles of NPFF at the spinal level.

NPFF has been referred to by several different names since its isolation. In order to minimize possible confusion during literature searches and reading primary references, we shall first clarify the terms used for NPFF in various studies. In the early studies, NPFF was often referred to as mammalian FMRF-NH_2-like peptide, FMRF-NH_2-like mammalian octapeptide or F-8-F-NH_2 because it was initially detected by an antiserum raised against FMRF-NH_2 and abbreviated as F-8-F-NH_2. When the peptide became commercially available, it was referred to as morphine modulating peptide or morphine modulating neuropeptide because it was sold by commercial sources under those names. In order to avoid confusion, the convention of naming peptides using the single letter amino acid abbreviations for their first and last amino acids was adopted and the name Neuropeptide FF (NPFF) was given and used since 1991. The wider family of peptides with Arg-Phe-amide at the C-terminus will be referred to collectively as RF-NH_2 peptides.

2. Analgesic, pronociceptive and morphine modulating activities of NPFF

NPFF has been reported to have pronociceptive and analgesic activities as well as pro- and anti-opioid effects. In general, i.c.v. injected NPFF exerts pronociceptive activity, while intrathecal injection induces analgesia [23,62]. The analgesic activity of NPFF may be an indirect effect of NPFF and will be further discussed later. These two opposite pharmacological activities have been thoroughly summarized and discussed previously [55,62]. However, two additional areas, the existence of other endogenous RF-NH_2 peptides and the modulatory role of RF-NH_2 peptides on acid sensing channels (ASICs), need to be considered in understanding the pharmacological activity of NPFF in pain processing.

Now it is clear that NPFF represents only one of numerous RF-NH_2 containing neuropeptides in mammalian nervous systems. Other RF-amide peptides identified so far include prolactin-releasing peptides (PrP_{20}, TPDINPAWYAGRGIRPVGRF-NH_2 and PrP_{31}) [25], metastin also referred to as Kisspeptin (Kisspeptin-13 [LPNYNWNSFGLRF-NH_2], Kisspeptin-14 and Kisspeptin-54) [39] and NPVF derived peptides SLNFEELKDWGPKNVIKMSTPAVNKMPHSFANLPLRF-NH_2 (human NPSF = RFRP-1) and VPNLPQRF-NH_2 (human NPVF) [44]. NPVF derived peptides have also been identified and referred to as RFamide-related peptides (RFRPs) in another study [26]. Among these RF-NH_2 peptides, PrP_{20} was suggested to be involved in nociception [43] and indeed was found to have analgesic activity when injected into the

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	Binding affinity NPFF-R1	$IC_{50} \pm$ S.D. NPFF-R2
hNPFF: SQAFLFQPQRF-NH_2	11.8 ± 3.3	1.1 ± 0.1
hNPAF: AGEGLSSPFWSLAAPQRF-NH_2	33.4 ± 9.3	3.3 ± 0.4
bNPFF: FLFQPQRF-NH_2	6.2 ± 1.2	3.1 ± 0.3
hNPSF-(1–37): SLNFEELKDWGPKNVIKMSTPAVNKMPHSFANLPLRF-NH_2	9.1 ± 1.3	7.5 ± 0.8
hNPSF-(26–37): MPHSFANLPLRF-NH_2	3.1 ± 0.6	6.1 ± 0.5
hNPVF: VPNLPQRF-NH_2	4.4 ± 0.5	122.3 ± 17.8

^{125}I-YANLPLRF-NH_2 and ^{125}I-YLFQPQRF-NH_2 were used as radioligands for competition assay. The data in the table are from Liu et al. (2001) [44]. The peptides derived from the human NPFF precursor are listed in the table because NPFF receptors studied in this table are of human origin. NPSFs in the table are predicted peptides from the NPVF precursor protein and are NPSF = RFRP-1 peptides.

parabrachial region of the dorsal medulla [34]. One of the RFRPs, NPSF = RFRP-1, was found to potently decrease morphine analgesia after i.c.v. administration [44]. Thus, it is possible that depending on the dose and location of injection, NPFF may exert the biological activity of other RF-NH_2 peptides. In fact, it has been suggested that the anti-opioid activity observed for i.c.v. injection of NPFF may be due to the ability of NPFF to exhibit the bioactivity of NPSF = RFRP-1; because NPSF = RFRF-1 is much more potent in attenuating mor-phine analgesia [44]. NPSF = RPRF-1 is an RF-NH_2 peptide with a strong affinity for NPFF-R1 which is much more widely distributed in brain than NPFF-R2 [44] judging from the distributions of NPFF-R2 mRNA and NPFF-R1 mRNA. More recently, another RF-NH_2 peptide precursor was predicted by a bioinformatic approach and subsequently cloned from human kidney cDNA and mouse spleen cDNA. One of the RF-NH_2 peptides, P518 (TSGPLGNLAEELN-GYSRKKGGFSFRF-NH2) deduced from the cloned precursor, was found to have high affinity for a G-protein coupled receptor SP9155 (also referred to as APR103) and display orexigenic activity [31]. Before the identification of peptide P518 as the ligand for SP9155, it had been suggested that NPFF and NPAF may also be ligands for the SP9155 GPCR [32]. The RF-NH_2 peptide, P518, was also identified by another study and referred to as 26RFa [15].

Following the isolation of NPFF and availability of NPFF antiserum [48], unlike many neuropeptides, the distribution of NPFF was found to be highly localized within the nervous and neuroendocrine systems. The highest levels of synthesis and storage occur in the dorsal spinal cord and posterior pituitary. Hence, it became clear that the spinal cord should be one of the most appropriate sites to study the bioactivity of NPFF. In contrast to i.c.v. injection, intrathecal administration of NPFF induced a long lasting analgesia in both thermal and mechanical tests [23]. Following this observation many studies have reported opioid like analgesic activity for intrathecally administered NPFF analogues [8,62,71,72]. This analgesic activity is less likely to be a direct effect of NPFF because it is long lasting (generally, injected small peptides undergo rapid enzymatic cleavage by proteases) and can be decreased by the opiate antagonist naloxone. In line with this observation, a functional interaction between NPFF and the enkephalin system at the spinal level was demonstrated: intrathecal infusion of the NPFF analogue 1DMe[D-Tyr^1, (NMe)Phe^3]NPFF produced a long lasting increase in spinal outflow of met-enkephalin-like immunoreactive material in the rat [9]. In addition, NPFF at subeffective doses was found to prolong the analgesic effect of morphine [23]. The mechanism underlying this observation is presently unclear. However, it is possible that NPFF, which shows no affinity for opioid receptors, may modify opioid receptor activity through some as yet undetermined mechanism. NPFF binding sites in primary afferent nerve terminals have been demonstrated using receptor binding autoradiography [21,22]. NPFF-R2 transcripts are expressed in both spinal cord (dorsal cord) and dorsal root ganglia (DRG) as revealed by the cloning of NPFF-R2 from the RNA extracted from DRG (unpublished result). Thus, it remains unclear whether the analgesic and/or morphine potentiating effect of intrathecally injected NPFF is mediated by activation of NPFF-R2 located in intrinsic dorsal spinal cord neurons, primary afferent terminals in the dorsal horn, or both.

Another possible action of NPFF or RF-NH_2 peptides in pain processing is at acid-sensitive ion channels (ASICs). ASICs (ASIC1, ASIC2b, and ASIC3) are expressed in the dorsal root ganglion (DRG) and predominantly in small diameter neurons [69]. ASICs are homo- and hetero-multimeric plasma membrane ion channels that respond to low pH. The role of ASIC channels in nociception and mechanosensation is strongly suggested by ASIC3 (DRASIC) knockout experiment [60] in which the knockout animals showed changes in responsiveness to noxious and innocuous mechanical stimuli and decreased responsiveness to noxious heat and acidic conditions. Various studies have reported that RF-NH_2 peptides delay the rate of ASIC desensitization thereby potentiating acid gated currents [7,14,17,70]. Furthermore, in mouse, intraplantar injection of the RF-NH_2 peptides, FMRF-NH_2 or KNFLRF-NH_2, was found to induce pain behavior as judged by licking of the injected paw [75]. Since FMRF-NH_2 and KNFLRF-NH_2 are not known to be endogenous peptides in mammalian systems, the possible functional RF-NH_2 peptides for modulation of ASICs remain to be determined. Using *Xenopus oocytes* co-expressing human or rat ASIC2A and ASIC3, the amplitude of the current induced by acidic solution was markedly increased by NPFF but to a lesser degree by FMRF-NH_2 [7]. Again using DRASIC expressed in *Xenopus oocytes,* NPFF potentiated the H^+ induced current [7]. Another RF-NH_2 peptide, NPSF (see Table 1) was found to be more potent than NPFF in potentiating proton gated currents in studies using cultured rat DRG neurons and ASICs expressed in COS cells [17]. NPVF derived peptides have also been shown to have modulatory activity on H^+-gated currents [70]. Interestingly, mRNA levels of ASIC1a, ASIC2b and ASIC3 in DRG were increased by Freund's adjuvant-induced inflammation [68] further supporting the role of ASIC in persistent pain states and the possible role of RF-amide peptides in nociceptive processing. The potential for NPFF to interact with ASICs in vivo is reinforced by the localization studies reviewed below.

3. Distribution of NPFF and its receptor(s) in the spinal cord

The distribution of NPFF in CNS has been studied by RIA [47] and also by immunohistochemistry [35,56]. A detailed localization of NPFF-immunoreactivity including NPFF positive neural pathways was extensively delineated [1,35,36,41,50,54]. Unlike other neuropeptides, NPFF is present in confined areas in the CNS with the highest levels in dorsal spinal cord and in the posterior lobe of the pituitary gland.

The highest level of NPFF was found in the dorsal spinal cord by radioimmunoassay (RIA) and, furthermore, the highest density of NPFF immunoreactive terminals and fibers was detected immunohistochemically in superficial laminae [5,47], an area important for pain processing. This network of NPFF terminals and fibers is mostly of intrinsic spinal origin [36,37]. NPVF derived peptides (human NPVF, VPNLPQRF-NH_2 and rat equivalent of NPVF, ANMEAGTMSHFPSLPQRF-NH_2 both recently identified [44,65]) share the identical C-terminal

tetrapeptide sequence PQRF-NH_2 with NPFF and also exist in spinal cord [66], thus they undoubtedly can complicate the interpretation of some of the early studies on NPFF peptides. The gene coding for the NPFF precursor protein was cloned [58,67] and one of the CNS regions found to have a high level of NPFF mRNA was the dorsal spinal cord [67,74]. In agreement with the analysis of hypothalamic NPFF immunoreactivity with HPLC followed by RIA, which showed that the immunoreactivity was biochemically distinct from authentic NPFF, transcripts for NPFF were not found in the intermediomedial nucleus in hypothalamus [67]. With the characterization of two genes coding for two structurally related peptides, NPFF and NPVF, the distributions of these two PQRF-NH_2 containing peptides can now be better differentiated. In the rat CNS, the distribution of NPFF mRNA is clearly different from that of NPVF mRNA. The highest levels of NPFF transcripts are in the spinal cord and medulla oblongata, which contains the trigeminal equivalent of the dorsal horn. In contrast, NPVF transcripts are most highly localized in the hypothalamus with a moderate level in the spinal cord [74].

In early studies using NPFF antiserum, absence of NPFF in spinal sensory ganglia was reported [56]. In the spinal cord, dorsal rhizotomy failed to decrease the NPFF level in dorsal spinal cord suggesting that the DRG is not a major source of dorsal spinal cord NPFF in normal rats [47]. In early studies, NPFF was not detected immunohistochemically in DRG [54,56]. NPFF mRNA was also reported to be absent from spinal ganglia using the method of in situ hybridization [67]. On the other hand, there are studies indicating the presence of NPFF in spinal sensory ganglia [4,29]. A very low level of NPFF was detected by a highly sensitive RIA and HPLC coupled with RIA in DRG extracts from colchicine-treated rats [4]. This finding was further confirmed by detection of NPFF immunoreactivity in neuronal cell bodies in DRG of colchicine treated rats. However, NPFF immunoreactivity was not found in DRG of normal rats in good agreement with earlier studies. Colchicine was used to block the axonal transport in order to accumulate NPFF immunoreactivity in neuronal cell bodies. The failure of rhizotomy to decrease the level of NPFF in dorsal spinal cord is clearly due to the low level of NPFF in DRG in comparison to spinal cord and masking of the loss by overlap with NPFF expressed by spinal cord neurons. In rat DRG, NPFF immunoreactivity was localized to small and intermediate size neurons suggesting their possible association with nociceptive fibers [4]. In agreement with this observation, we have detected NPFF mRNA in DRG of both rat and human and also in rat DRG cell cultures [29]. The discrepancy between the in situ hybridization studies and RT-PCR results may be explained by the very high sensitivity of RT-PCR. Furthermore, we found that NPFF gene expression in DRG was regulated by peripheral inflammation [29]. This observation will be discussed further later.

NPFF specific binding sites were demonstrated in rat spinal cord membranes [2], and this binding was reduced by guanine nucleotides suggesting a G-protein coupled receptor for NPFF [57]. Autoradiographic studies have demonstrated that NPFF binding sites are widely distributed in the brain and spinal cord [6]. The regions found to have a high density of binding sites include the superficial layer of dorsal spinal cord and trigeminal tract, regions relevant for nociceptive sensory transmission and processing. Subsequently, G-protein coupled receptors with high affinity and specificity for NPFF and NPAF were identified, characterized and referred to as NPFF receptor 1 (NPFF-R1) and NPFF receptor 2 (NPFF-R2) [12,20]. In the rat, the distribution of NPFF-R2 gene expression is very similar to that of NPFF immunoreactivity; both NPFF-R2 mRNA and NPFF are highly localized in the superficial layer of dorsal spinal cord as shown in Fig. 2 compared with much lower levels in other regions of the CNS [12,44,74]. High–affinity binding sites for NPFF were also demonstrated in postmortem specimens of human spinal cord with the highest density in the superficial layer of the dorsal horn using [^{125}I]YLFQPQRF-NH_2 [3]. This high density of NPFF binding sites may partially be due to NPFF-R1 because NPFF-R1 is expressed abundantly in human spinal cord. Though, NPFF-R2 mRNA is in much lower abundance in the human

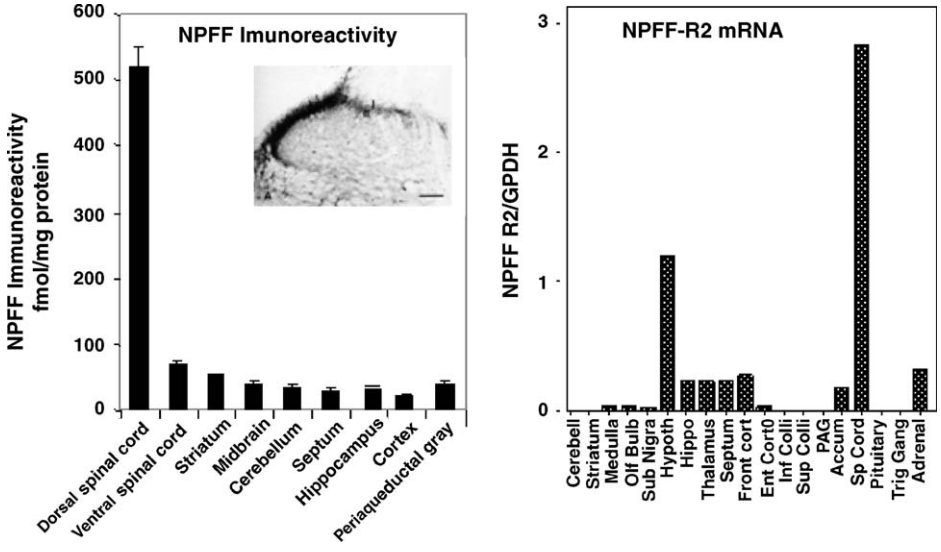

Fig. 2 – Distribution of NPFF Immunoreactivity (left) [47] and NPFF-R2 mRNA in rat CNS (right) [74]. The insert shows the immonohistochemical localization of NPFF in the rat spinal cord [56].

spinal cord in comparison with the rat, it is readily detected in the human dorsal spinal cord by RT-PCR [74]. Immunohistochemical studies using an antibody raised against human NPFF-R2, has revealed dense NPFF-R2 immunoreactivity in the superficial layers of dorsal spinal cord and in spinal trigeminal nucleus of the African green monkey and this distribution pattern is further confirmed with in situ hybridization and autoradiographic studies. From this study, it has been inferred that human NPFF-R2 may have a modulator role in sensory processing at the spinal level [76]. In rat spinal cord, both NPFF and NPFF-R2 mRNA are also detected around lamina X.

Several lines of evidence suggest that NPFF receptors are present on primary afferent terminals of the rat dorsal spinal cord. An early quantitative autoradiographic study reported that only a slight but non-significant decrease of [^{125}I]YLFQPQRF-NH_2 binding was observed in the superficial layers of the rat spinal cord following neonatal capsaicin treatment or rhizotomy [45], results that suggest NPFF binding sites are not located on primary afferent terminals. In contrast, another autoradiographic study using [^{125}I][D.Tyr^1, (NMe)Phe^3]NPFF has reported a significant decrease of binding sites in the superficial layers of the spinal cord by neonatal capsaicin treatment, dorsal rhizotomy or sciatic nerve transection suggesting that a portion of NPFF binding sites in the dorsal spinal cord are located on the primary afferent terminals [21]. The discrepancy in the above two studies is suggested to be due to the fact that [^{125}I][D.Tyr^1, (NMe)-Phe^3]NPFF is much more stable than [^{125}I]YLFQPQRF-NH_2. It may be also interesting to question whether these two radioligands bind different receptors. Our immunohistochemical study, with an antibody raised against NPFF-R2, on the spinal cord revealed that some of the dorsal spinal cord NPFF-R2 originated from dorsal root ganglia in the rat as revealed following peripheral inflammation [29]. NPFF-R2 mRNA was detected by RT-PCR of total RNA extracted from dorsal root ganglia or trigeminal ganglia though in much lower level in comparison with dorsal spinal cord [74]. NPFF-R2 mRNA was shown to be non-detectable in trigeminal ganglia (Fig. 2) under the conditions used for RT-PCR analysis; however, NPFF-R2 mRNA was clearly detected when the number of PCR cycle was increased [74,29]. It is very likely that in the normal rat, NPFF-R2 in the dorsal spinal cord is preferentially located in intrinsic spinal cord neurons; however, the functional role of dorsal spinal cord NPFF receptor originating from DRG cannot be ignored especially in chronic pain or persistent inflammatory conditions. We have also detected NPFF-R2 mRNA in human dorsal root ganglia suggesting the relevance of studying the DRG NPFF-R2 mRNA in the rat model of pain [29] and reinforcing a potential role for the NPFF system in human pain processing.

4. Regulation of NPFF and its receptors in animal models of inflammatory pain

The effect of peripheral inflammation on spinal NPFF was studied in rats with carrageenan inflammation of the hind paw [38]. Immunohistochemically, NPFF positive neuronal cell bodies were not observed in the spinal cords of normal rats [5,37] but were only detected in the spinal cord of rats treated intraspinally with colchicine to block axonal transport [37]. In contrast, during peripheral inflammation, NPFF positive neurons were detected in the dorsal spinal cord on the side ipsilateral to the inflammation without colchicine treatment [38]. The results suggest that NPFF biosynthesis and, by extension NPFF function, may be up-regulated at the spinal level by inflammatory pain. Following the cloning of the gene encoding the NPFF precursor protein, regulation of NPFF mRNA expression was studied by in situ hybridization in the rat [67]. The number of NPFF mRNA-expressing cells in the spinal cord was demonstrated to be increased by hind paw inflammation induced by intraplanter injection of carrageenan, while no change in NPFF mRNA expression was observed in the spinal cord of rats with the spinal nerve tightly ligated using the Kim and Chung neuropathic pain model. The difference in nociception-inducing mechanisms in the carrageenan and spinal ligation models was suggested as the explanation for these dissimilar results. The recently identified human NPVF (also known as RFRP-3) [26,44] is identical to NPFF in its C-terminal tetrapeptide sequence, PQRF-NH_2. They are both present in dorsal spinal cord and the functional role of NPVF is unknown. Because of this, regulation of NPVF and NPFF gene expressions was also examined and compared. Up-regulation of gene expression by carrageenan inflammation was detected by RT-PCR for NPFF but not for NPVF [74]. The results suggest that the spinal NPFF system is involved in pain mechanisms and the structurally similar NPVF derived peptides may have different function(s).

To further explore the role of the NPFF system in pain processing we have studied the regulation of the two NPFF receptors in the spinal cord of rats with peripheral inflammation induced by carrageenan [74]. Levels of NPFF-R2 transcripts in the rat dorsal spinal cord were increased by carrageenan inflammation of the hind paw [74]. This result is consistent with the previous study reporting an increased ^{125}I-1-dimethyl-YLFQPQRF-NH_2 binding in the spinal cord of rats with joint inflammation induced by *Mycobacterium butyricum* suspended in Freund's adjuvant injected into the tibio-tarsal joint [46]. NPFF-R1 mRNA, present in much lower abundance than NPFF-R2 mRNA in the rat spinal cord, was not affected by the peripheral inflammation [74]. Interestingly, affinity and specificity studies of the NPFF-R2 as well as the corresponding distributions of NPFF-R2 and NPFF (Fig. 2) seem to suggest that NPFF-R2 rather than NPFF-R1 is the functional receptor for NPFF [12,44,74]. The strong expression of both the peptide and mRNA components for the NPFF precursor and NPFF-R2 in spinal cord is shown in Fig. 2.

Regulation of NPFF and NPFF-R2 mRNA was also studied recently in different pain models, acute colonic inflammation, inflammatory pain and neuropathic pain, in the rat [53]. Expression of NPFF mRNA and NPFF-R2 mRNA was not affected in the spinal cord but NPFF-R2 was slightly elevated in the brain stem region in the acute colonic inflammation model. In the neuropathic pain model induced by tight ligation of a spinal nerve, again gene expression of NPFF and NPFF-R2 was not altered at the spinal cord level. In contrast to these two models, both NPFF and NPFF-R2 gene expressions were significantly increased in the spinal cord by the carrageenan

Table 3 – Summary on regulation of NPFF and NPFF receptors at the spinal level

Treatment	Effects at spinal level
Carrageenan 0.2 mg injected into hind paw	↑ of NPFFir cell bodies in spinal cord [38]
Carrageenan 0.2 mg injected into hind paw	↑ of NPFF mRNA in spinal cord [67]
Tight ligation of spinal nerve	↔ of NPFF mRNA in spinal cord [67]
Carrageenan 6.0 mg injected into hind paw	↑ of NPFF mRNA in spinal cord [74]
Colitis by TNBS instilled into colon	↔ of NPFF mRNA in spinal cord [53]
Carrageenan 0.2 mg injected into hind paw	↑ of NPFF mRNA in spinal cord [53]
Tight ligation of spinal nerve	↔ of NPFF mRNA in spinal cord [53]
Mycobacterium butyricum	↑ of ^{125}I-1-dimethyl-YLFQPQRF-NH_2
in Freund's adjuvant injected into tibio-tarsal joint	binding sites in spinal cord [46]
Carrageenan 6.0 mg injected into hind paw	↑ of NPFF-R2 mRNA in spinal cord [74] and DRG [29]
Carrageenan 6.0 mg injected into hind paw	↔ of NPFF-R1 mRNA in spinal cord [74]
Carrageenan 6.0 mg injected into hind paw	↑ of NPFF-R2ir in primary afferent terminals [29]
Carrageenan 0.2 mg injected into hind paw	↑ of NPFF-R2 mRNA in spinal cord [53]
Tight ligation of spinal nerve	↔ of NPFF-R2 mRNA in spinal cord [53]

Increased and unchanged are indicated by ↑ and ↔, respectively. NPFFir = NPFF immunoreactivity and NPFF-R2ir = NPFF-R2 immunoreactivity.

induced inflammation of the hind paw, a result consistent with our earlier study [74].

Contradictory results have been reported for the localization of the NPFF system in spinal sensory ganglia as discussed in the third section on distribution of NPFF and its receptor(s) in the spinal cord. In our recent study, we have demonstrated the presence of NPFF mRNA in the rat DRG and, furthermore, an up-regulation of NPFF gene expression induced by peripheral inflammation following carrageenan injection into the hind paw. To study the distribution of NPFF-R2 and the effect of peripheral inflammation on the NPFF-R2 in the spinal cord, an antiserum against NPFF-R2 was generated. The NPFF-R2 immunoreactivity was found in laminae I and II, in laminae V, VI and X. Following unilateral hind paw inflammation, an additional NPFF-R2 immunoreactivity was localized to axonal bundles just above and within lamina I [29]. This distribution suggests that a portion of the increased NPFF-R2 immunoreactivity is located on primary afferent terminals. To confirm this up-regulation, NPFF-R2 mRNA was analyzed in the DRG by RT-PCR and found to be increased by hind paw inflammation induced by carrageenan [29]. Our studies on the DRG suggest that the NPFF system in the spinal sensory ganglia may also be involved in pain processing. Although the physiological consequences of up-regulation of the spinal NPFF system during peripheral inflammation still remain to be explored, in view of the long lasting morphine enhancing activity of intrathecally injected NPFF, it is possible that the spinal NPFF system may contribute to the increased spinal analgesic potency of morphine in animals with peripheral inflammation [28,61]. In the rat, it was found that i.t. and i.c.v. administered NPFF (at doses higher than the amount used to induce analgesia and morphine modulating activity in normal rats [23]) attenuated allodynia in rats with chronic inflammation induced by complete Freund's adjuvant or neuropathic pain induced by fitting a 2-mm-long cuff of polyethylene tubing (slit longitudinally) around the sciatic nerve. In contrast, no effect was found for NPFF when injected i.t. into normal rats [8]. These observations suggest that in the inflammatory model (induced by complete Freund's adjuvant), the up-regulated spinal NPFF-R2 mRNA may account for the allodynia-modulating activity of NPFF. A similar antinociceptive activity was found for intrathecally injected [D-Tyr^1,(NMe)Phe^3]NPFF, an NPFF analogue, in CCI induced mononeuropathic and streptozocin induced diabetic rats but not in normal rats [16] using a paw pressure test. This antinociceptive activity of [D-Tyr^1,(NMe)Phe^3]NPFF was found to involve μ- and δ-opioid receptors. In terms of gene regulation, NPFF-R2 gene expression was not altered in the neuropathic pain model induced by tight ligation of sciatic nerve [53]; whether there is a regulation of NPFF-R2 mRNA at the spinal cord level in the neuropathic pain model induced by the polyethylene cuff or loose ligature of sciatic nerve (CCI) remains to be studied.

The animal model studies, summarized in Table 3, clearly indicate that the spinal NPFF system is involved in the neuronal pathways activated by inflammation or inflammatory pain. The analgesic activity of intrathecally administered NPFF or its analogues was found to be more pronounced in rats with inflammatory or neuropathic pain than in normal rats [8,16]. Though these observations raise a possible involvement of NPFF system in neuropathic pain, the regulation of NPFF system in various pain models still needed to be examined. While the exact role of NPFF or its receptor(s) requires additional investigation, the identification of the multiple sites of NPFF and NPFF-R2 expression in the primary afferent-spinal cord circuit will aid in clarifying the role this system plays in nociceptive modulation.

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