Expression and regulation of two RFamide peptide genes in the rat

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Academic dissertation

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To my husband Jari

and

our daughter Saana

Contents

Abstract 6
List of original publications 7
List of abbreviations 8
1. Review of the literature 9
1.1 General aspects of neuropeptides 9
1.2 The RFamide family of neuropeptides 11
1.3 Biosynthesis and secretion of neuropeptides 13
1.4 Neuropeptide receptors 15
1.5 Functional properties of neuropeptides 17
1.5.1 Energy and fluid homeostasis 17
1.5.2 Neuropeptides and prolactin secretion 19
1.5.3 Cardiovascular control 19
1.5.4 Neuropeptides, adrenal hormones and the immune system 21
1.5.5 Neuropeptides and ingestion 22
1.6 Role of neuropeptides in the development 23
1.7 Mechanisms of pain and involvement of neuropeptides 24
1.7.1 Pain circuits 25
1.7.2 Pain modulatory pathways 26
1.7.3 Pain and RFamides 27

2. Aims of the study 29

3. Materials and methods 30
3.1 Ethics 30
3.2 Animals 30
3.3 Experimental models 30
3.3.1 Hyperosmolar stimulus 30
3.3.2 Methods for assessing pain 31
3.3.3 Injections 31
3.4 Cloning 31
3.5 Immunohistochemistry 32
3.6 In situ hybridisation 32
3.7 Measurements and statistics 33
3.7.1 Measurements 33
3.7.2 Quantification of gene expression 33

3.7.3 Statistics 34

4. Summary of results 35
4.1 Structure of the NPFF gene 35
4.2 NPFF and PrRP gene expression in the rat tissues 36
4.2.1 Central nervous system 36
4.2.2 Peripheral tissues 38
4.3 Embryonal mRNA distribution of NPFF and PrRP 39
4.3.1 Central nervous system 39
4.3.2 Peripheral tissues 39
4.4. Effect of hyperosmolar stimulus on NPFF and PrRP gene expression 40
4.5. NPFF and PrRP in pain models 41
4.5.1 Neuropathic pain 41
4.5.2 Inflammatory pain 42

5. Discussion 43
5.1 RFamide peptide gene structure, expression and regulation 43
5.2 RFamide peptide genes in development 43
5.3 Functional significance of RFamide peptides 44
5.3.1 Body fluid homeostasis and RFamide peptides 44
5.3.2 RFamides and peripheral functions 45
5.3.3 PrRP, NPFF, and pain 45
5.4 Expression of the UHR1/GPR10 -like receptor 47
5.5 Methodological considerations 47

6. Summary and conclusions 49

7. Acknowledgements 50

8. References 52

Abstract

The classical concept of neuropeptides considers them as peptides that act on nervous tissue and either are co-expressed with classical transmitters or act as hormones. It later became clear, however, that neuropeptides are in fact found throughout the body and have effects also on peripheral tissues. Neuropeptides typically have numerous functions and may have hormone-like effects. They are classified by structure, function or site of expression. The precursor molecules are large and often multiple copies of the same peptide, or several distinct, biochemically active peptides may be spliced from the same precursor. Neuropeptides act through and bind to multiple types of receptors mediating diverse functions.

The RFamide (R=arginine, F=phenylalanine) peptides are a group of neuropeptides that share the C-terminal RF-NH_2 –structure. Neuropeptide FF was the first one characterized among these peptides, and our aim was to clone the gene for the peptide precursor and show the mRNA distribution in normal and stimulated situations. During the preparation of this work, other researchers found several other RFamides. The prolactin-releasing peptide was the second RFamide and was chosen to be studied in parallel with neuropeptide FF to reveal possible similarities and differences between the two peptides.

Both peptides occur in the medulla and hypothalamus though in distinct nuclei: e.g., PrRP in the caudal, and NPFF in the rostral division of the nucleus of the solitary tract. Only neuropeptide FF occurs in the spinal cord. According to our findings, the appearance of the peptide mRNAs during embryonal development is distinct and suggests that from early developmental stages, the roles of these peptides differ. Both peptides occur in a limited number of peripheral tissues (NPFF only in the spleen, PrRP in male reproductive organs, kidney, and liver) and are involved in nociceptive modulation: the prolactin-releasing peptide through a medullary mechanism (analgesic through nucleus of the solitary tract and hyperalgesic through caudal ventrolateral medulla) and the neuropeptide FF through both medullary (antiallodynic) and spinal (analgesic) mechanisms. Neuropeptide FF, but not prolactin-releasing peptide mRNA, is regulated by chronic hyperosmotic stimulus.

In conclusion, these two neuropeptides, although occurring in the central nervous system in adjacent areas, obviously have distinct functions. In addition, these results suggest a possible new mechanism for the transcriptional regulation of neuropeptide FF.

List of original publications:

The experiments and conclusions in this thesis are based on the following original communications, referred to in the text as Roman numerals I to IV. In addition, some unpublished results are presented.

I Vilim F.S., Aarnisalo A.A., *Nieminen M.-L.,* Lintunen M., Karlstedt K., Kontinen V.K., Kalso E., States B., Panula P. and Ziff E. Gene for pain modulatory neuropeptide FF: induction in spinal cord by noxious stimuli. Mol. Pharmacol. 1999; 55(5): 804-811.

II *Kalliomäki M.-L.* and Panula P. Neuropeptide FF, but not prolactin-releasing peptide, mRNA is differentially regulated in the hypothalamic and medullary neurons after salt loading. Submitted.

III *Nieminen M.-L.,* Nystedt J. and Panula P. Expression of neuropeptide FF, prolactin-releasing peptide and the receptor UHR1/GPR10 genes during embryogenesis in the rat. Dev. Dyn. 2003; 226: 561-569.

IV *Nieminen M.-L.,* Brandt A., Pietilä P., Panula P. Expression of mammalian RFamide peptides neuropeptide FF (NPFF), prolactin-releasing peptide (PrRP) and the PrRP receptor in the peripheral tissues of the rat. Peptides 2000; 21:1695-1701.

List of abbreviations

ACTH	adrenocorticotropin
bp	base pairs (of the gene sequence)
cAMP	cyclic adenosine mono phosphate
cDNA	complementary deoxyribonucleic acid
cGMP	cyclic guanylyl mono phosphate
E1	embryonal day 1
FMRFamide	molluscan neuropeptide with phenylalanine-methionine-arginine-phenylalanine amino acid sequence at the carboxyterminus
HPLC	high-pressure liquid chromatography
i.v.	intravenous
mRNA	messenger ribonucleic acid
MSH	melanocyte-stimulating hormone
NPAF	neuropeptide AF, AGEGLSSFWSLAAPQRF-NH_2
NPFF	neuropeptide FF, FLFQPQRF-NH_2
NPSF	neuropeptide SF, SLAAPQRF-NH_2
NPY	neuropeptide Y
OXY	oxytocin
P0	postnatal day 0
PIP	phosphatidyl inositol 4,5-bisphosphate
POMC	pro-opiomelanocortin
PPTA	preprotachykinin B
PPTB	preprotachykinin A
PrRP	prolactin-releasing peptide
RER	rough endoplasmic reticulum
RFamide	mammalian neuropeptide corresponding to molluscan FMRFamide
RT-PCR	reverse transcriptase polymerase chain reaction
SP	substance P
VIP	vasoactive intestinal peptide
VP	vasopressin

Amino acids and their symbols:

A	alanine
C	cysteine
D	aspartic acid
E	glutamic acid
F	phenylalanine
G	glycine
H	histidine
I	isoleucine
K	lysine
L	leucine
M	methionine
N	asparagine
P	proline
Q	glutamine
R	arginine
S	serine
T	threonine
V	valine
W	tryptophan
Y	tyrosine

1. Review of the literature

1.1 General aspects of neuropeptides

Neuropeptides (De Wied et al. 1969, Kastin et al. 1979) are small proteins in neurons mostly co-expressed with classical transmitters or hormones (Hökfelt 1991, Lundberg 1996). They are expressed throughout the nervous system and are also present in a variety of peripheral tissues. Once they were thought to act on neural tissues only, but nowadays their role is understood as an integrating tool between the peripheral organs and nervous tissue. Neuropeptides act as neurohormones, as neurotransmitters, and as neuromodulators. The hormones are regarded as agents mediating their effects at long distances through the circulation, and the transmitters as affecting only locally the nearby (paracrine) or the same (autocrine) cells. These neurotransmitters must, by definition, be synthesized in the presynaptic terminals and be released by them upon nerve stimulation. A neuromodulator is traditionally understood as an agent that does not evoke an effect on its own, but alters the responses induced by a classical transmitter pre- or postsynaptically. Some of the main properties of neurotransmitters and neuromodulators are considered in Table 1, in which corresponding properties of neuropeptides are compared, as well.

Table 1. Properties of neurotransmitters, neuromodulators, and neuropeptides. (Modified from Strand: Neuropeptides: regulators of physiological processes, 1999, MIT Press and (Hökfelt et al. 2000). EPSP=excitatory postsynaptic potential, IPSP=inhibitory postsynaptic potential, RER=rough endoplasmic reticulum.

FUNCTION	NEUROTRANSMITTER	NEUROMODULATOR	NEUROPEPTIDE
Release	Induced by high Ca^{2+} influx	Dependent on action potential, intermittent/continuous	Caused by small fluctuations in Ca^{2+}
Reuptake mechanisms	Active	No	No, but degraded in the intercellular space
Localization	Near the synapses	Not restricted to the synapse	Not restricted to the synapse
Synthesis	In the soma, but also in the nerve endings	-	Only ribosomal de novo synthesis, axonal transportation to the nerve endings
Transportation	Directly to the mature secretory granules	-	Through the RER to secretory granules
Coexistence	Can be found alone in the vesicles	With transmitters	With transmitters or alone
Structure	Constant	May vary and as consequence of biological activity	-
Potency	Low	High	High
Effect on postsynaptic membrane	Excitatory (EPSP) and inhibitory potential (IPSP)	Affects postsynaptic membrane, but incapable of evoking EPSP or IPSP: modulates transmitters	Affects postsynaptic membrane, but incapable of evoking EPSP or IPSP: modulates transmitters

One should keep in mind, however, that the definition of an agent should be considered site-specifically, e.g., substance P fulfills the criteria of a neurotransmitter in the spinal cord, but modifies the action of acetylcholine on its receptor in the spinal ganglia.

Most commonly, neuropeptides are designated according to a structure in their sequence (e.g., neuropeptide Y) or to their first-discovered function (e.g., gastrin-releasing peptide), even though they are usually later shown to be involved in various functions. First, the neuropeptides were divided into groups according to site of origin, but this became difficult, since the peptides are found in many organs. An attempt to classify some important neuropeptides in the families appears in Table 2.

The peptide precursor molecules are usually large, containing one or more copies of the same peptide and/or a related peptide with possibly other expression patterns and functions. For example, the pro-opiomelanocortin (POMC) contains several bioactive peptide sequences: α-, β-, and γ-melanocyte-stimulating hormone (MSH) and in additon β-lipoprotein, adrenocorticotropin (ACTH), and β-endorphin. The existence of several copies of the same peptide in the precursors provides evidence of gene duplication. During evolution, minor changes or attributions in gene duplication have occurred, resulting in slightly different sequences of the same peptide in different species. Acher (1980) described the evolution of vasopressin and oxytocin: They evolved from the same ancestral precursor. The first gene duplication resulted in the generation of two distinct peptides with different activities: vasopressin and oxytocin. The next duplications caused minor sequence differences between teleosts, amphibians, reptiles, and mammals. Even though the peptide is highly homologous, the receptors usually prove to have less homology: The vasopressin is identical in the rat and human being, but the receptors have only 50% homology (Hoyle 1999).

Large, hydrophobic, and high molecular-weight molecules such as peptides have difficulty in penetrating the blood-brain barrier. Some peptides may, however, cross the blood-brain barrier by transmembrane diffusion (Banks and Kastin 1985) related to peptide hydrogen bonding (Chikhale et al. 1994), as does vasopressin, or by saturable bi- (Barrera et al. 1991) or unidirectional (Barrera et al. 1987) transport systems. Peptides are also capable of altering the blood-brain barrier permeability to other substances: Melanocortins can alter the tight junctions or passive membrane diffusion and thus facilitate the passage of some i.v.-introduced radiolabeled agents that are generally considered markers of blood-brain barrier integrity (Rudman and Kutner 1978).

Table 2. Examples of neuropeptide families. See list of abbreviations for amino acid symbols.

FAMILY	PEPTIDES AND ABBREVIATIONS	STRUCTURAL SIMILARITY
Bombesin-like peptides	Bombesin Gastrin-releasing peptide GRP Neuromedin B NMB Rantesin	C-terminal WAVGHXM
Endogenous opiates	Proenkephalin Prodynorphin Pro-opiomelanocortin POMC	3 genes: overall similarity, similar size and position of introns, 6 cysteines near N-terminus
Calcitonin gene-related peptides	Calcitonin CT Calcitonin gene-related peptide CGRP	CGRP developed by alternative splicing from CT
Cholecystokinin	Cholecystokinin CCK Gastrin	C-terminal GWDMF
Hypothalamic peptides	Oxytocin OXY Vasopressin VP	CYXQNCPXG-NH_2
Hypothalamic releasing peptides	Corticotropin-releasing hormone CRH Growth hormone-releasing hormone GHRH Luteinizing hormone-releasing hormone LHRH Thyrotropin-releasing hormone TRH	-
Insulin-like growth factors	Insulin Insulin-like growth factors Relaxin	Similar primary and tertiary structures
Neurotensin	Neuromedin Neurotensin NT	C-terminal PYIL
Pancreatic polypeptides	Neuropeptide Y NPY Pancreatic polypeptide PP Peptide YY PYY	Similar structure of the precursor. 24% homologous active peptides
RFamide peptides	KiSS-peptides KiSS Neuropeptide FF NPFF Neuropeptide SF NPSF Prolactin-releasing peptide PrRP P518 RFRPs RFRP	C-terminal RF-NH_2
Somatostatin	Somatostatin SS	-
Somatotropins	Choriomammotropin PL Growth hormone GH Prolactin PRL	Ancestral prototype-GH-PRL. >90% similarity of coding regions
Tachykinins	Neurokinin A NKA Neurokinin B NKB Substance P SP Bradykinin	C-terminal FXGLM
VIP-secretin-glugacon-family	Gastric inhibitory peptide GIP Glucagon Pituitary adenylate cyclase-activating peptide PACAP Secretin Vasoactive intestinal peptide	Similar structural organization of the genes

1.2 The RFamide family of neuropeptides

Currently five genes are known to encode several RFamide peptides in mammals (Perry et al. 1997, Hinuma et al. 1998, Hinuma et al. 2000, Kotani et al. 2001a, Liu et al. 2001, Jiang et al. 2003). In invertebrates, over 20 peptides have been characterized. FMRFamide peptides are encoded in genes that contain multiple copies of active peptides in invertebrates. The mammalian FMRF-crossreactive peptides are larger, and their precursor contains only one copy of the peptide, each of which contains C-terminal glycines. The first RFamide peptide recognized was FMRFamide, a cardioexcitatory peptide in mollusks (Price

and Greenberg 1977), also involved in modulation of opiate-induced analgesia (Tang et al. 1984). Yang et al. (1985a) isolated from bovine brain in 1985 by HPLC-purification two FMRF-NH_2 crossreacting peptides, FLFQPQRF-NH_2 and AGEGLSSPFWSLAAPQRF-NH_2. These two neuropeptides are derived from the same precursor. The second RFamide peptide, the prolactin-releasing peptide, was found in 1998 as a ligand for an orphan G protein-coupled receptor UHR1/GPR10-like receptor (Hinuma et al. 1998). Majane and Yang (1987) —since their HPLC experiments revealed in the brain other immunoreactive materials in addition to FLFQPQRF-NH_2 and AGEGLSSPFWSLAAPQRF-NH_2 —found evidence of several related neuropeptides. Immunohistochemical data (Panula et al. 1996) also showed specific FLFQPQRF-NH_2 -immunofluorescent staining in a cell group between the dorsomedial and ventromedial hypothalamus, which in later studies appeared to express another neuropeptide (Maruyama et al. 1999, Minami et al. 1999).

Indeed, soon another RFamide peptide was characterized as a ligand for an orphan receptor expressed in the hypothalamus (Hinuma et al. 1998). This peptide was capable of releasing prolactin, and was designated prolactin-releasing peptide, PrRP. The preproprotein of PrRP also contains two possible sequences for mature peptides, SRAHQHXMEIRTPDINPAXYAGRGIRPVG and TPDINPAWYAGRGIRPVGR, called PrRP31 and PrRP20, respectively. The PrRP20 may be a cleaved form of PrRP31. Three TATA boxes exist in the gene, the one located at –92bp being crucial for gene expression. The PrRP gene contains three exons and two introns, the second intron being located precisely at the cleavage site of PrRP20 from PrRP31. Exon 1 encodes the 5´region; exon 2 starts the sequence of PrRP31; the last exon encodes for the rest of the coding sequence, comprising PrRP20 and the 3´untranslated region. (Yamada et al. 2001). The structural organization of the gene is shown in Figure 5 on page 37.

Two groups searched the human genome databases independently and succeeded in isolating an unknown peptide precursor encoding for two mammalian peptides: RFRP-1 (MPHSFANLPLRF-NH2) and RFRP-3 (VPNLPQRF) or NPSF and NPVF (Hinuma et al. 2000, Fukusumi et al. 2001). High homology appeared between human, rat, mouse, bovine, and quail genes (Hinuma et al. 2000, Liu et al. 2001). The C-terminal amino acids of hRFRP-3 are identical to those of NPFF, and the four C-terminal amino acids of hRFRP-1 coincide with the chicken LPLRF-NH2 (Dockray et al. 1983), suggesting for the peptides a common evolutionary ancestor. The *in situ* hybridization studies of these peptides revealed peptide mRNA and immunoreactivity in the hypothalamus near the third ventricle between the dorsomedial and ventromedial nuclei (Hinuma et al. 2000, Fukusumi et al. 2001), exactly where NPFF immunoreactivity had been evident.

The third new peptide precursor, KiSS-1 (Lee et al. 1996, Lee and Welch 1997) encodes for four kisspeptins: -10, -13, -14, and –54, numbers indicating the number of amino

acid residues in the corresponding peptide. The sequence of kisspeptin–54 (also called metastin) is flanked by basic residues, which indicates that the peptide is cleaved from the precursor by furin or prohormone convertases. The shorter fragments are most likely degradation products of the kisspeptin-54 (GTSLSPPPESSGSRQQPGLSAPHSRQIPAPQGAVLVQREKDLPNYNWNSFGLRF-NH2). The kisspeptins were isolated from human placenta and were shown to be capable of releasing oxytocin (Kotani et al. 2001a). KiSS-1 gene expression has been detected most abundantly in the placenta, but also in the testis, pancreas, liver (Ohtaki et al. 2001), and brain (Muir et al. 2001).

Recently, Jiang et al. (2003), using a novel computational approach, succeeded in finding a novel ligand for an orphan G protein-coupled receptor SP9155. This ligand, called P518, shares the RF terminus with these peptides and is expressed in the brain, coronary arteries, thyroid and parathyroid glands, testes, prostate, large intestine, and colon of the human and the mouse. The precursor of this peptide also contains multiple putative bioactive peptides.

All currently known RFamide peptides are listed in Figure 1.

```
NPAF                          AGEGLSSPFWSLAAPQRF-NH2  bovine            1
NPAF human                    AGEGLNSPFWSLAAPQRF-NH2  human             1
NPSF                                 SLAAPQRF-NH2  bovine, human     1
NPFF                                 FLFQPQRF-NH2  bovine, human     1
GnIH-RP-2                      SSIQSLLNLPQRF-NH2  quail             2
FGRP                            SLKPAANLPLRF-NH2  frog              3
RFRP-2                          SAGATANLPLRS-NH2  bovine            4
RFRP-1                          MPHSFANLPLRF-NH2  bovine            4
RFRP-3                               VPNLPQRF-NH2  bovine            4
GnIH                            SIKPSAYLPLRF-NH2  quail             5
LPLRFamide                               LPLRF-NH2  chicken           6
RFRP                          ANMEAGTMSHFPSLPQRF-NH2  rat               7
PrRP20                      TPDINPAWYAGRGIRPVGRF-NH2  bovine            8
PrRP31     SRAHQHSMEIRTPDINPAWYAGRGIRPVGRF-NH2  bovine            8
C-RFa                       SPEIDPFWYVGRGVRPIGRF-NH2  carp              9
KiSS-14                             DLPNYNWNSFGLRF-NH2  human, rat       10
P518             TSPGLGNLAEELNGYSRKKGGFSFRF-NH2  human            11
```

Figure 1. Comparison of RFamide peptides. Residues identical in at least three of the sequences are shaded. The number at the end of the line refers to the original publication as follows: 1) Yang et al. 1985, 2) Satake et al. 2001, 3) Koda et al. 2002, 4) Hinuma et al. 2000, 5) Tsutsui et al. 2000, 6) Dockray et al. 1983, 7) Ukena et al. 2002, 8) Hinuma et al. 1998, 9) Fisher et al. 1997, 10) Kotani et al. 2001, 11) Jiang et al. 2003.

1.3 Biosynthesis and secretion of neuropeptides

Neuropeptide precursors are large, often containing sequences of more than one bioactive peptide. Preprohormones contain the precursor molecule of a peptide and an N-terminal signal sequence that guides the preprohormone through the rough endoplasmic reticulum (RER), which is an ATP-dependent process. The signal peptide is removed in the RER and reacts with the RER to form a tunnel facilitating the translocation of the peptide chain through the RER (Blobel and Dobberstein 1975a, 1975b). Protein folding, disulfide formation, and N-glycosylation occur in the endoplasmic reticulum (Mains et al. 1987). The biologically inert

prohormone is transferred to the *cis*-face of the Golgi apparatus and from there on to the *trans*-Golgi network, where it is packaged into secretory granules. The neuropeptide precursor molecules are located in large, dense core vesicles or secretory granules together with the prohormone convertases and other enzymes and usually some other transmitter. Sometimes the precursor undergoes post-translational proteolysis before entrance into the vesicles, in which case, the products of the same precursors remain in distinct granules. Usually a classical transmitter, e.g., acetylcholine or noradrenaline, is coexpressed in the neuron or even in the same vesicle as the neuropeptide (Hökfelt et al. 1984, 1986). Before excretion, the precursors are cleaved to bioactive peptides, possibly phosphorylated, O-glycosylated, amidated, acetylated, or sulphated (Mains et al. 1987, Seidah and Chretien 1997, Steiner 1998). A simplified scheme of peptide biosynthesis and secretion is presented in Figure 2.

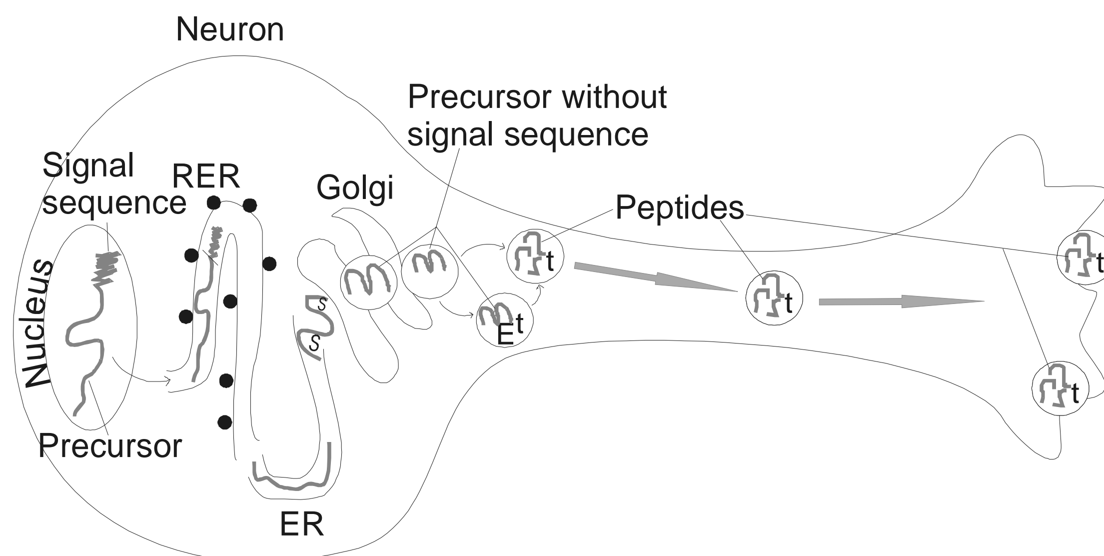

Figure 2. Peptide biosynthesis and secretion in a neuronal cell. RER=rough endoplasmic reticulum, ER= endoplasmic reticulum, t= classical transmitter, E=peptide processing enzyme, S=disulfide bridge.

The release of various agents from the nerve terminal and the response they cause are dependent on the stimulus of the cell and on the receptors on the postsynaptic membrane. The effects of simultaneously excreted agents can be either synergistic or opposing. The nerve cells in the same organ may contain different combinations of neuropeptides: e.g., substance P is expressed in dorsal root ganglion neurons, and some of these neurons may contain dynorphin in addition to SP, while some may contain other neuropeptides. There are no publications indicating or denying the coexistence of RFamides with neurotransmitters.

Once released, the neuropeptides are most often degraded in the extracellular space by both specific and non-specific peptidases. The peptidases are numerous, and most of them act on several peptides. In a number of neuropeptides, carboxypeptidases remove C-

terminal basic residues. Aminopeptidases remove the N-terminal amino acids from precursor molecules (Lowther and Matthews 2002). Other peptidase families are vasopeptidases and metallopeptidases, which contain a zinc motif. Problems in peptidase activity may lead to clinically significant problems such as obesity when the carboxypeptidase E is deficient, leading to a severe defect in proinsulin conversion (Naggert et al. 1995). Moreover, peptidase inhibitors may also be useful in treating illnesses such as hypertension with vasopeptidase (angiotensin-converting enzyme and neutral endopeptidase) inhibitors (Campbell 2003). Neuropeptides are ineffective when introduced orally, being rapidly degraded in the gastrointestinal tract.

The knowledge of RFamide biosynthesis is very limited; it can only be stated that C-terminal glycine residues required for amidation exist in the precursor (Vilim and Ziff 1995).

1.4 Neuropeptide receptors

Neuropeptides bind to several types of receptors activating different second messenger systems. In addition, the neuropeptides may alter the affinity of a receptor to its specific ligand. They bind most commonly to the G protein-coupled receptors, but bind other types of receptors, as well. G protein-coupled receptors have seven transmembrane helices and activate cyclic adenosine monophosphate (cAMP), phosphatidyl inositol 4,5-bisphosphate (PIP), or calcium ions (Ca^{2+}) as second messengers. For example, the oxytocin-vasopressin peptide family binds to four receptors, OXY receptor and V_{1A}-, V_{1B}-and V_2-receptors. The OXY receptor is selective for oxytocin over vasopressin, and the rest preferentially bind vasopressin. The V_2-receptor activates adenylate cyclase and increases the amount of cAMP; all the other receptors activate phospholipase C and activate the PIP pathway (Hoyle 1999). Additional diversity in neuropeptide effects results from the fact that receptors may couple to different kinds of G-proteins (inhibiting or activating), and the effect is the calculated sum of all the second messenger systems (Fuxe et al. 1995). Guanylyl cyclase is an intracellular enzyme that can be membrane-bound or free in the cytosol (Wedel and Garbers 1997). It activates cGMP, a compound similar to cAMP, which contains guanosine instead of adenosine. The membrane-bound guanylyl cyclase acts as a receptor for atrial natriuretic peptide, and the free guanylyl cyclase is activated by nitric oxide and free radicals. Neuropeptides, e.g., FMRFamides (Bowman et al. 1995), interact with nitric oxide, and may thus modulate the free guanylyl pathway. Other types of receptors are the one transmembrane helix-containing tyrosine-kinase receptors, cytokine receptors, and ion channels. Insulin and most growth factors bind to tyrosine kinase-coupled receptors that in the cytoplasm initiate a cascade of protein phosphorylations. These receptors are constituted of α- and β-chains forming the trans-, intra-, and extracellular domains that contain cysteine-rich domains (Ullrich et al. 1986). Cytokine-receptor type I receptors bind prolactin and

growth hormone and activate Janus Kinase (JAK) or the signal transducer and activator of transcription (Stats).

Neuropeptide receptor types are schematized in Figure 3.

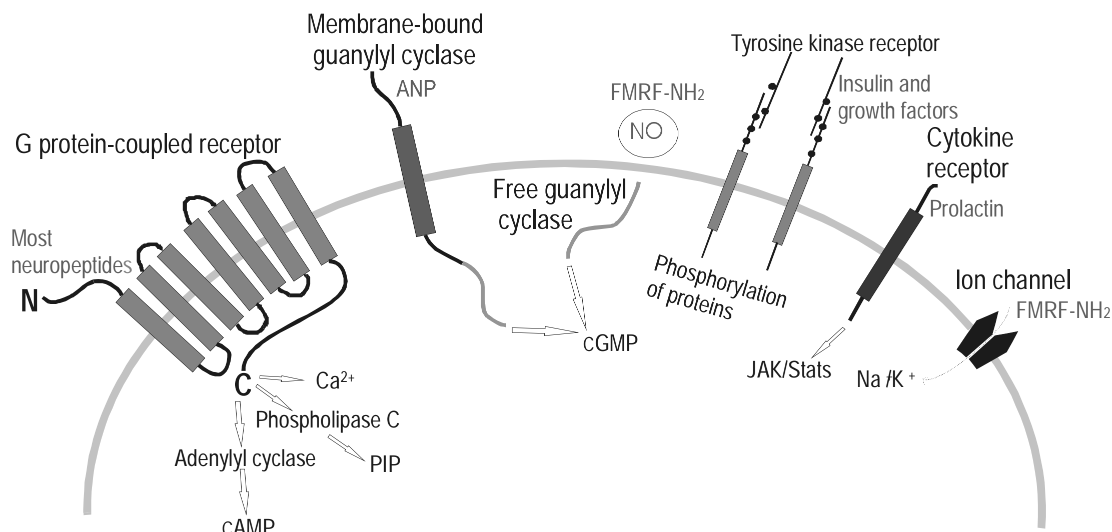

Figure 3. Scheme representing different types of receptors activated by neuropeptides in different organs and cells. ANP=atrial natriuretic peptide, NO=nitric oxide, JAK/Stats=Janus kinase/signal transducer and activator of transcription.

The FMRFamides bind both to G protein-coupled receptors (Higgins et al. 1978) and ion channels (Lingueglia et al. 1995). Study of RFamide-peptide receptors in mammals is currently somewhat confusing. For example, the same receptor OT7T022 has been proposed as a receptor both for deduced RFRP:s (Hinuma et al. 2000) and for NPFF, called the NPFF1 receptor (Bonini et al. 2000). On the other hand, the C-terminal portion of the peptide sequence accounts for the binding of the peptide to its receptor (Mazarguil et al. 2001, Kotani et al. 2001b), and RFRP and NPFF share the last four amino acids at their C-terminal ends; non-amidated forms are inactive (Kotani et al. 2001b).

In addition to cardiovascular regulation and hormone-like effects, NPFF possesses both anti- and pro-opioid functions, which has complicated the search for a specific receptor. Elshourbagy et al. (2000) isolated a 1888kb cDNA encoding a 420 amino acid protein resembling orexin A receptor (Sakurai et al. 1998). They tried more than one thousand ligands and found that the selective ligands all had RF-termini and bound only to cells transfected with both HLWAR77 and a promiscuous G protein α subunit. NPFF and NPAF were the most potent ligands, with EC50 values of 13 ± 5 and 22 ± 6 nmol. Similarly, (Bonini et al. 2000) show that NPFF activates the G protein- coupled receptors NPFF1 and NPFF2. These receptors are abundant in human tissues, NPFF1 especially in the spinal cord and NPFF2 in the placenta. Mostly, NPFF1 is found in the CNS. Its distribution is quite different in the rat, which may be explained by differing effects of the peptide among species. In addition

to activation G protein-coupled receptors, NPFF may bind to acid-sensing cation channels (Askwith et al. 2000), of which the human heteromeric subtype ASIC2A+3 is mainly responsible for these effects (Catarsi et al. 2001).

All four kisspeptins bind with similar affinity and efficacy to GPR54, a rat receptor structurally related to neuropeptide receptors, in particular to galanine receptors (28-40% identity) (Kotani et al. 2001a, Muir et al. 2001, Ohtaki et al. 2001). Three publications described simultaneously an orphan receptor that binds the kisspeptins that are expressed in the placenta and brain. Sequences verified that the AXOR12 (Muir et al. 2001) and h/rOT7T175 (Ohtaki et al. 2001) are orthologues of GPR54, the term used in this dissertation. The GPR54 seems to be coupled to G_q class proteins, since pertussis toxin has no effect on Ca release (Muir et al. 2001,Kotani et al. 2001a). As for most G-protein coupled receptors, arachidonic acid release is stimulated and ERK½ and p38 pathways are activated by GPR54 (Kotani et al. 2001a). GPR54 is expressed in placenta, pituitary, lymphocytes, pancreas, adipose tissue, and brain (amygdala, n. accumbens, hippocampus, cingulate gyrus) (Muir et al. 2001).

Prolactin-releasing peptide was first characterized as a ligand for a G protein-coupled receptor UHR1/GPR10-like receptor (Hinuma et al. 1998). This PrRP receptor gene contains two exons, one intron, and two polyadenylation signals. Two major and one minor transcriptional start-sites exist at 139, 140bp upstream, and –161bp from the translational start-site. The promoter region contains several putative binding sites for transcriptional factors, but obviously no typical TATA or CAAT box (Ozawa et al. 2002); in their publication, they also show that the UHR1/GPR10-like receptor is upregulated in pituitary adenomas and downregulated by treatment with bromocriptine, a D2-agonist, which means that the PrRP receptor gene is possibly regulated by dopamine.

1.5 Functional properties of neuropeptides

1.5.1 Energy and fluid homeostasis

The center for energy balance in the brain is the hypothalamus. It contains several nuclei that express neuropeptides taking part in food and water balance. The essential nuclei are the arcuate nucleus at the basal hypothalamus just above the median eminence, the paraventricular nucleus in the anterior hypothalamus bilaterally to the uppermost third ventricle, and the supraoptic nucleus anterior and lateral to the median eminence. The ventromedial hypothalamic nucleus is considered to be the satiety center, and the lateral hypothalamic nucleus to be the feeding center. The most prominent neuropeptides involved in feeding and satiety regulation are NPY, orexins, and galanin. Neuropeptides involved in reducing food intake are cholecystokinin and α-MSH. NPY in the arcuate nucleus is inhibited

by insulin and leptin, and when functional problems occur in these neuropeptides, the experimental animals become obese. (Williams et al. 2001).

Body fluid balance is also controlled by the hypothalamic paraventricular and supraoptic nuclei that project into the neurohypophysis. Neurohypophyseal hormones originate from the hypothalamus (Palkovits 1982). As to neuropeptides, the two most prominent are vasopressin (VP) and oxytocin (OXY), but also NPFF immunoreactivity exists in the neurohypophysis. VP and OXY are synthesized in the magnocellular (VP also in the parvocellular) neurons of the paraventricular and the supraoptic nuclei in the hypothalamus, but rarely in the same cell. The VP- and OXY-containing magnocellular neurons send axons into the neurohypophysis and spinal cord and brainstem, and perform their para- and autocrine regulation through the soma and dendrites in the paraventricular and supraoptic nuclei. Their precursors contain neurophysin I (OXY) and neurophysin II (VP). Neurophysins are cleaved before the formation of the active neuropeptides, but they remain in the same secretory granules with OXY and VP (Acher and Chauvet 1995).

Oxytocin is controlled by and involved in female reproductive functions such as coitus, parturition, lactation, puberty and development, circadian rhythm, and plasma osmolality (Burbach et al. 1992). Vasopressin is mostly known for its involvement in body fluid homeostasis and vascular tone. Because vasopressin mRNA expression is upregulated due to hyperosmotic stimuli (Valtin et al. 1965, Majzoub et al. 1983), Brattleboro rats lacking the functional vasopressin gene develop diabetes insipidus, an inability to concentrate their urine (Schmale and Richter 1984, Schmale et al. 1984).

NPFF concentration is high in the pituitary and is located in nerve terminal-like structures (Kivipelto et al. 1989). Indeed, NPFF is co-regulated with vasopression in the neurohypophysis (Majane and Yang 1990, Majane and Yang 1991). NPFF cannot be detected in the pituitary of the Brattleboro rat (Majane and Yang 1990), and the amount of NPFF immunoreactive material in the heterozyogous animals was diminished, as was the amount of vasopressin. Due to a hyperosmotic stimulus, hypophyseal NPFF and vasopressin peptide levels are reduced equally (Majane and Yang 1991). Additional evidence exists that NPFF affects vasopressin levels: Immunoglobulin G fraction from antiserum against NPFF increases vasopressin release in rats (Yokoi et al. 1998). Intracerebroventricular injections of NPFF attenuate vasopressin release, and food intake is diminished (Arima et al. 1996, Murase et al. 1996, Yokoi et al. 1998, Sunter et al. 2001).

The endocrine pancreas contains four types of cells (α-, β-, δ-, and PP-cells) that express and secrete glucagon, insulin, somatostatin, and pancreatic polypeptide. Insulin is anabolic, increasing the storage of glucose into the liver, and glucagon stimulates hepatic glycogenolysis, gluconeogenesis, and ketogenesis. Somatostatin acts in a paracrine manner and inhibits the secretion of both insulin and glucagon. The degree of release of insulin is

dependent upon the states of activity of various nerve terminals and the phase of the insulin-secreting β-cell (Ahren 1999). The nerve terminals around the pancreatic islets contain both classical neurotransmitters and at least two neuropeptides, vasoactive intestinal peptide and pituitary adenylate cyclase-activating peptide (PACAP). (Havel et al. 1997, Filipsson et al. 1999). NPFF inhibits both prolactin and somatostatin release from the pancreatic islets (Fehmann et al. 1990), but because no peptide immunoreactivity has been isolated from the pancreas, it may be speculated that its effects are mediated via the blood circulation.

1.5.2 Neuropeptides and prolactin secretion

Prolactin is a hormone secreted from the anterior pituitary to induce lactation. It was long thought that the secretion of prolactin is controlled merely by the inhibitory action of dopamine and somatostatin, although researchers were keenly searching for a prolactin-releasing factor. Endogenous opioids, e.g., β-endorphin (Yang et al. 2000), are reported to be involved in prolactin release in stimulated situations such as lactation (Arbogast and Voogt 1998) and mating (Yang et al. 2000). Thyrotropin-releasing hormone (at 100 nM, *in vitro*) and vasoactive intestinal peptide are examples of neuropeptides capable of inducing prolactin release, although high doses are required. Both NPFF (at 3 and 30 nM, *in vitro*) and PrRP (100 nM-1μM, *in vitro*) have some prolactin-releasing properties (Aarnisalo et al. 1997, Hinuma et al. 1998), but both are more potent in other systems such as cardiovascular control (Roth et al. 1987, Samson et al. 2000) and modulation of pain (Roumy and Zajac 1998).

1.5.3 Cardiovascular control

Blood pressure and heart rate are centrally controlled by the cardiovascular center in the medulla and its connections to the hypothalamus and autonomic nervous system. Various neuropeptides are found in these locations, and many of them are indeed involved in cardiovascular regulation. NPY is one of the peptides that participates in medullary cardiovascular control through medial nucleus of the solitary tract, together with catecholamines and excitatory amino acids (Glass et al. 2002). Vasopressin and oxytocin inhabit most of the hypothalamic paraventricular and supraoptic nuclei and are obviously both involved in cardiovascular regulation directly or through other mediators such as atrial natriuretic peptide and nitric oxide (Coote 1995, Cunningham et al. 2002, Petersson 2002).

Peptides from the same family may possess completely different properties in cardiovascular regulation: endogenous opiates are involved in a controversial manner; γ_2-MSH may be either bradycardic and depressive or tachycardic (at the dose 50-100 nmol/kg) and pressor (at the dose 50 nmol/kg), and the other MSHs do not affect blood pressure or

heart rate (Klein et al. 1985, de Wildt et al. 1993). The receptor for the actions of γ_2-MSH is yet to be identified; it is not necessarily a melanocortin receptor but may also be a receptor that binds the RFterminus responsible for the cardiovascular effects, such as a NPFF receptor or an FMRFamide receptor (Versteeg et al. 1998). ACTH, another derivative of the POMC precursor, produces a depressor effect when administered i.v. (at the dose 15-500 nmol/kg) (Van Bergen et al. 1997, Nakamura et al. 1976), but is clearly advantageous in improving blood pressure and pulse amplitude in hemorrhagic shock (at the dose 160 μg/kg) (Bertolini et al. 1986).

Intravenous NPFF elevates blood pressure, at the dose 15μg/kg (5.5 nmol/kg), in rats (Roth et al. 1987). It is found in the central nervous system in areas involved in blood pressure regulation, such as the medulla (nucleus of the solitary tract) and hypothalamus (Kivipelto et al. 1989), being colocalized with catecholamines (Kivipelto et al. 1992). Allard et al. (1995) proposed that the cardiovascular effects of NPFF may also be mediated by peripheral mechanisms after revealing NPFF binding sites and NPFF-ir in the heart. Some of the cardiovascular effects of intravenous NPFF may, however, be mediated by postganglionic neuronal components, since hexamethonium, a ganglionic blocker, partly reduces the cardiovascular effects of NPFF. This is in accordance with the results from a study performed by Laguzzi et al. (1996), revealing the involvement of the dorsal vagal complex in the blood pressure-regulating effects of NPFF. Allard et al. (1995) showed also that NPFF could act as a catecholamine modulator. NPFF did not alter catecholamine levels, but the effects of NPFF were attenuated in catecholamine-depleted rats: α_1- and β_1 – antagonists partially reversed intravenously injected NPFF-induced augmentation in blood pressure. On the other hand, α_2-antagonists enhanced the pressor and cardioexcitatory effects of NPFF. Prazosin, an α_1-antagonist, also inhibits the increase in blood pressure induced by injections into the nucleus of the solitary tract (Laguzzi et al. 1996). Bilateral injections of NPFF into this nucleus provoke a rise in blood pressure and a decrease in heart rate, indicating involvement in cardiovascular regulation of the central reflex. Another RFamide, the PrRP, has also been shown to increase blood pressure; the doses required are, however, large (0.4-4.0 nmol) compared to for example angiotensin (0.1 nmol) (Samson et al. 2000).

Further convincing evidence that NPFF is involved in central cardiovascular regulation is that the NPFF neurons in the nucleus of the solitary tract, and those projecting into the pontine parabrachial nucleus, are biochemically activated (c-Fos expression is induced) in rats after a cardiovascular challenge, this effect being more prominent following hypotensive stimulation than after hypertensive stimulation (Jhamandas et al. 1998). The development of the putative NPFF agonist and antagonist, PFRFamide and PFR(Tic)amide,

respectively (Huang et al. 2000), was thought to be helpful to the investigation of cardiovascular effects of NPFF. However, the pharmacological properties of these agents do not fully correspond to those of NPFF and need further investigation.

Like the cardiovascular effect of NPFF (Allard et al. 1995, Huang et al. 2000), PrRP also causes hypertension when injected intracerebroventricularly, although heart rate was not measured, and the potency of intravenous injection was not tested. PrRP does not seem to alter water- or salt appetite as opposed to other peptides known to affect blood pressure (Samson et al. 2000). Taking into account that PrRP has a binding site in the heart and is also found in plasma (Matsumoto et al. 1999), it is possible that it also has peripheral cardiovascular properties.

1.5.4 Neuropeptides, adrenal hormones, and the immune system

The hypothalamus funnels information from several brain areas (e.g., sensory cortex, limbic system, medulla oblongata) and transforms it to hormonal signals. For example, corticotropin-releasing hormone, CR, regulates the output of ACTH from adenohypophysis, which in turn regulates the adrenocortical secretion of glucocorticoids. The adrenal medulla secretes catecholamines, noradrenaline, adrenaline, and dopamine. Enkephalins are part of the family of endogenous opioids, and of these methionine-enkephalin (Met-enk), is colocalized with adrenaline in the adrenal medulla, where other neuropeptides such as SP are also present in the adrenal chromaffin cells. Sympathetic activation increases medullary discharge. The adrenal cortex secretes mineralo- and glucocorticoids, and their secretion is controlled centrally by the hypothalamus-pituitary system and by changes in plasma ion concentration. Adrenal medullary cells may, however, affect adrenocortical secretion in a paracrine manner (Nussdorfer 1996). Evidence of NPFF immunoreactivity in the adrenal gland is controversial. Labrouche et al. (Labrouche et al. 1998) attested to the presence of NPFF immunoreactivity in both adrenal medullary and cortical cells, in areas where NPY is found. Our NPFF antibodies fail to bind to the adrenal medulla (Lee et al. 1993, Panula et al. 1996). It is possible that the antibody Labrouche et al. used crossreacts with either NPY or with a neuropeptide from the RFamide family.

Neuropeptides, e.g., corticotropin-releasing hormone and ACTH, act directly in stimulating glucocorticoids that modify immune reactions and indirectly by regulating the central nervous system that then generates nerve impulses regulating the immune response. Inflammatory processes enhance production of leucocytes, and several neuropeptides are strongly involved in inflammation such as SP, which is proinflammatory and somatostatin, which is anti-inflammatory. (Berczi et al. 1996). Many neuropeptides are expressed by the immune cells themselves. The amounts produced are lower than in neuronoendocrine cells, but lymphocytes are far more numerous, and thus the plasma levels of neuropeptides

secreted are sufficient (Clarke and Bost 1989). The neuropeptides may be immunostimulatory (αMSH), immunosuppressive (PRL), or immunomodulatory (endorphins, VP, OXY, SP) in nature. Neuropeptides such as SP, NPFF (stimulates at a dose 0.1 pM, and inhibits at a dose 0.1μM) (Lecron et al. 1992), and somatostin may affect lymphoid cell proliferation or may modulate antibody responses (MSH, SP) or production (opiates). Taken together, neuropeptides play a regulatory role, despite the low levels of secretion, in immune homeostasis, disturbances of which lead to serious autoimmune and allergic diseases (Berczi et al. 1996).

1.5.5 Neuropeptides and ingestion

Many neuropeptides occur in the epithelium or nervous system of the gastrointestinal tract and have various hormone-like effects (Table 3). The neuropeptide-secreting cells in the gut are of ectodermal origin, not of neural crest origin, as are those peptide-secreting cells in the adrenal medulla and autonomic ganglia.

Table 3. Cells secreting neuropeptides in the gastrointestinal tract.

	LOCATION:	**NEUROPEPTIDES:**	**FUNCTION:**
Endocrine cells	Colon	SP	Increase motility
Enterochromaffin cells	Whole GI-tract	SP, enkephalin	Increase motility
Enteroglucagon cells	Ileum	Glucagons	-
A cells	Stomach	Glucagons	Stimulate liver glycogenolysis
D cells	Stomach fundus	Somatostatin	Inhibit secretion of other hormones
G cells	Stomach antrum	gastrin17	Stimulate secretion and motility
I cells	Duodenum and jejunum	Cholecystokinin	Cause gall-bladder contraction and secretion of pancreatic juice
Immune cells (blood derived or resident)	Basal lamina of GI-tract	SP	-
K cells	Duodenum and jejunum	GIP	Inhibit gastric secretion and motility
M cells	Duodenum and jejunum	Motilin	Affect interdigestive motility
N cells	Ileum	Neurotensin	Inhibit motility and increases ileal blood flow
S cells	Duodenum and jejunum	Secretin	Increase secretion of bicarbonate
Enteric neurons	Gut walls	SP, NKA, NPY, dynorphin, met-enkephalin	Enhance motility and secretion

The intestines are responsible for digestion of food both chemically (secretions of the stomach, duodenum, and exocrine pancreas) and mechanically (peristalsis). Neuropeptides are involved in these processes through both central and peripheral regulation. Central regulation involves, for example, emotions that cause responses such as diarrhea during stress, and constipation in depressive patients. Peripheral regulation consists of actions of the enteric nervous and autonomic systems within the bowel walls. The main transmitters are

acetylcholine and noradrenaline, but these are often colocalized with neuropeptides. All sphincters involved in the gastrointestinal tract are also regulated by neuropeptides.

NPFF and FMRFamide affect gut contractibility (Raffa and Jacoby 1989, Demichel et al. 1993, Decker et al. 1997). The effect of NPFF on the intestines is somewhat contradictory, since it seems to block morphine-induced inhibition of bowel movements in the ileum (at doses of 10 nmol-1 μmol) (Demichel et al., 1993) but to enhance it in the colon (at 13 nmol) (Raffa and Jacoby 1989). NPFF affects feeding behavior also through the CNS; intracerebroventricular injections of NPFF (5-10μg) reduce food intake (Murase et al. 1996). Thus far no evidence links NPFF to the cells of the gastrointestinal tract, and its effects on regulation of food ingestion and bowel movements must thus be mediated by the blood. NPFF has been detected in human plasma. Measured by radioimmunoassay, the quantity of NPFF, 2.9 pg/ml, is insufficient for systemic action, in the light of the K values of NPFF receptors being 0.06 nmol (Sundblom et al. 1995).

1.6 Role of neuropeptides in development

The gene expression of neuropeptides arises at different time-points, and neuropeptides play various roles in organ development. Opioid peptides (met-enkephalin, β-endorphin, and dynorphin) appear in the brain at embryonal day (E) 11.5 and their receptors later: the μ-receptor at E12.5, κ-receptor at E14.5, and δ-receptor at postnatal day (P) 0 (Rius et al. 1991). Somatostatin, neurotensin, and SP immunoreactivities are also apparent during the early phases of development (Shiosaka et al. 1981a and b, Hara et al. 1982, Inagaki et al. 1982, Sakanaka et al. 1982). Some peptides become measurable later during the embryonal period, like vasopressin at E16 in the hypothalamus but only at E19 in the pituitary (Buijs et al. 1980).

Neuropeptides may have growth-enhancing or -restricting properties during development. Zagon et al. (1999) showed that the met-enkephalin (10 mg/kg) acts through the ζ-opioid receptor to inhibit growth in all three embryonal layers (excluding lungs and bones) and that this inhibition is reversed by naltrexone. Met-enkephalin and its precursor, proenkephalin A, are expressed in embryonal cells at E20. During early development, vasoactive intestinal peptide agonists induce postimplantational growth, and antagonists reduce growth (Gressens et al. 1993 and 1994). VIP mRNA is lacking from the mouse embryo at this critical period (E9-11), although binding sites and immuoreactivity are present. Radioactively labeled VIP injected i.v. into the pregnant female was detected in the mouse conceptuses at E10 (Spong et al. 1999). It is thus obvious that VIP of maternal origin is at least in part responsible for postimplantational growth during embryogenesis. Insulin-like

growth factors are involved in the regulation and development of several organs such as the liver (Streck et al. 1992), placenta (Zhou and Bondy 1992), and neurons (Anlar et al. 1999).

Of all the RFamide peptides only NPFF has undergone study during development of rat the CNS from E16 to P28 (Kivipelto et al. 1991). NPFF immunoreactivity appears first at E20 in the median eminence as fibers and then disperses on the first postnatal day to the nucleus of the solitary tract, to the septal area, nucleus accumbens, stria terminalis, retrochiasmatic area, hypothalamus, central gray, inferior colliculus, and parabrachial nucleus. In the spinal cord dorsal horn, NPFF immunoreactivity is first apparent on P3. The immunoreactive signal strengthens until the age of 2 weeks. From P21 to P28 appear the highest number of NPFF-immunoreactive neurons and fibers, and their distribution corresponds to that of the adult rat.

1.7 Mechanisms of pain and involvement of neuropeptides

In human beings, pain is subjective, but can be measured by visual analogy scales (Huskisson 1974). The sensation of pain is a consequence of a stimulus strong enough to cause tissue damage, and its intensity is proportional to the intensity of the stimulus. Responses vary, however, according to psychological states or due to sensitization of the sensory afferents (Beecher 1965, Melzack et al. 1982).

Investigation of sensitivity to noxious stimuli can be studied in animals only postnatally. Studies in embryos have concentrated on reflex responses to somatic stimuli, reflexes starting in human fetuses at 7.5 weeks of gestation (Bradley and Mistretta 1975) and at E15 in the rat (Narayanan et al. 1971), at time-points when spontaneous movements of the fetus begin (Narayanan et al. 1971, de Vries et al. 1982). The first area to become sensitive is the mouth region and the last the tips of the toes (E19) (Reynolds et al. 1991) and the tail region. At birth the reflexes still involve whole-body movement, and the cutaneous reflexes are exaggerated in comparison to those of adults (Ekholm 1967, Stelzner 1971, Issler and Stephens 1983). The somatosensory reflexes begin to change at birth to more localized reactions.

Pain may be classified as acute, chronic, inflammatory, or neuropathic. Acute pain consists of painful stimulation causing the activation of pain fibers by chemical mediators such as prostaglandin and bradykinin. If the sensation becomes prolonged without new external stimulation, the pain sensation is said to be chronic. Inflammation is represented by rubor (redness), tumor (swelling), calor (heat), and dolor (pain). Inflammatory markers are many; SP, for instance, has been shown to cause all the typical signs of inflammation, and antagonists to SP to completely block the symptoms in humans (Lotz et al. 1988, Murthy et al. 1991). Neuropeptides e.g., cholecystokinin (Wiesenfeld-Hallin et al. 2002), galanin (Flatters et al. 2003), and NPY (Ossipov et al. 2002), are also involved in neuropathic pain

that originates from nerve injury, a pain that has traditionally been very difficult to treat in patients, although some have benefited from antidepressant therapy. Interestingly, SP depleters (agents that selectively activate C-fibers and cause the release of SP, like capsaicin, and thus inhibit the transmission of the painful signal to higher brain areas) have proven to be effective when applied topically (Rains and Bryson 1995, Lipman 1996). SP-inhibitors acting centrally may also prove to be potential pain killers, although there is controversy as to their true potency (Monck 2001, Brune 2002).

1.7.1 Pain circuits

The primary afferent neurons are A and C fibers innervating the muscles, joints, skin, and viscera. They sense thermal (>44°C), mechanical, chemical, and polymodal pain. The primary sensory afferent fibers enter the central nervous system via the spinal cord dorsal horn, where the information is processed by intrinsic dorsal horn neurons. These give rise to tracts (spinothalamic, spinoreticular, spinomesencephalic, spinocervical, and postsynaptic dorsal column) that convey information to the brain. Each tract usually crosses to the opposite side of the spinal cord before reaching its destination and making contact with the neurons extending to the higher brain areas. The spinothalamic tract seems to be essential for pain (Mayer et al. 1975, Simone et al. 1991). The projection neurons in general are involved in controlling the information transferred along the spinal cord by central inhibitory pathways (Schaible et al. 1991). The dorsal horn also contains propriospinal neurons and local interneurons. Propriospinal neurons convey information along the spinal cord from one segment to another, but their role in the sensation of pain is poorly understood. Local interneurons transfer information only short distances inside the spinal cord (Figure 4).

Neuropeptides are found in the spinal cord, and some examples are described here. Substance P is found in the primary afferents bringing information to the spinal cord laminae I, II, and V (Hökfelt et al. 1975), and SP is released in the spinal cord upon noxious stimulation (Duggan et al. 1988). In the rat's ipsilateral paw, SP receptors are activated and internalized in the dorsal horn lamina I deep and superficial neurons in response to thermal and mechanical stimulation after inflammation (Abbadie et al. 1997). Propriospinal pathways also contain substance P (Pickel et al. 1983). NPFF is located in the spinal cord laminae I, II, and X at all levels (Kivipelto and Panula 1991). NPFF and NPY are likely to be located in the local interneurons, since in the spinal cord rhizotomy does not affect NPFF/NPY content (Kivipelto and Panula 1991). Enkephalins also occur in the interneurons (Cuello 1983). No evidence exists of descending tracks containing NPFF (Kivipelto and Panula 1991), but some NPY-containing neurons in the spinal cord arise from the brainstem in addition to arising from the spinal cord itself (Minson et al. 2001).

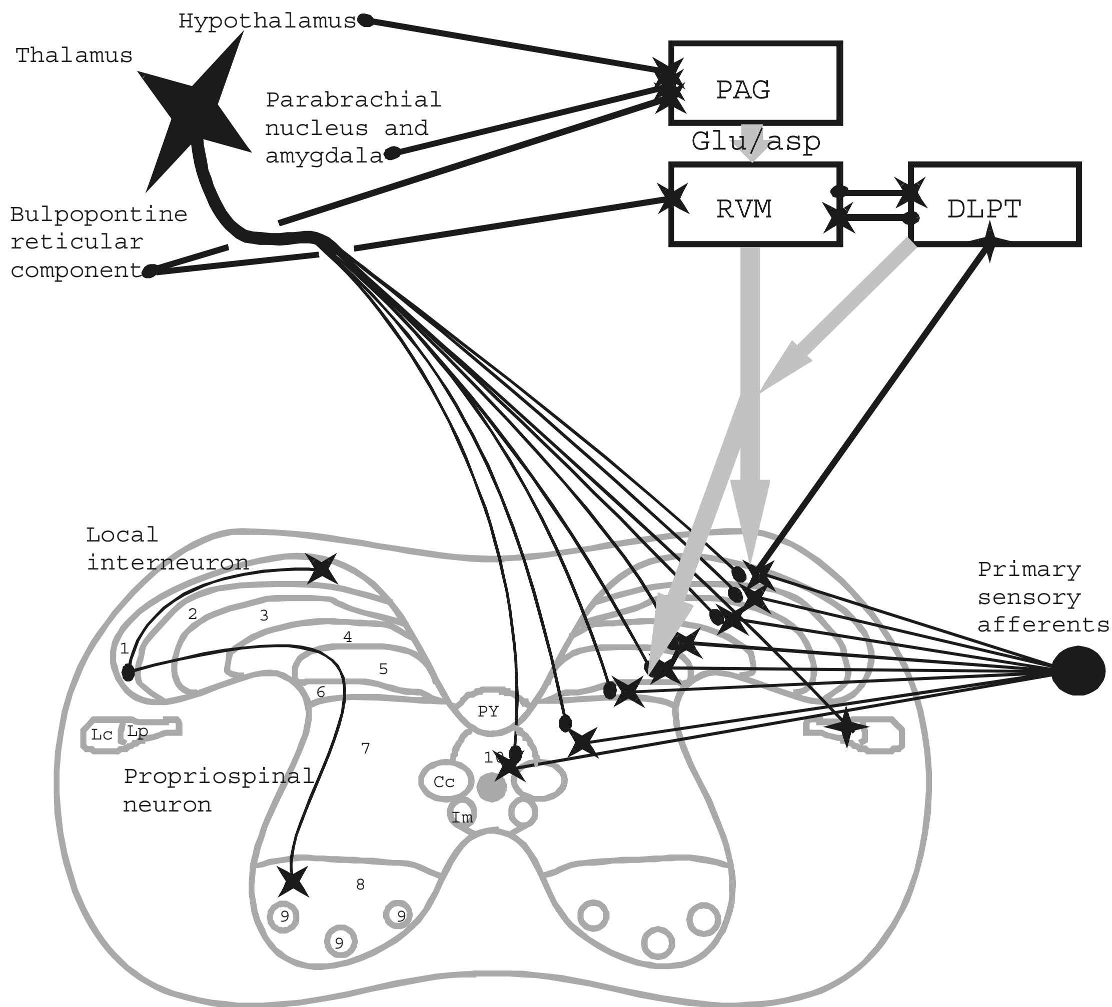

Figure 4. Structure of and pain modulation in the spinal cord. Thin lines represent neurons of the spinal cord and primary sensory neurons bringing information from the periphery via C-, Aδ- and Aβ-fibers. Semi-thin lines represent neurons conveying pain modulatory information in higher brain areas. Star and thick line schematizes the tracts conveying information from spinal cord to brain. Origins of neurons are marked with black ovals and synapses with small stars. Gray arrows show pain modulatory pathways from upper brain regions to the spinal cord. Abbreviations: 1-9=spinal cord laminae, Cc=central cervical nucleus, DLPT=dorsolateral pontomecenphalic tegmentum, Im=intermediolateral column, Lc=lateral cervical nucleus, Lp=lateral spinal nucleus, PAG=periaqueductal gray, PY=pyramidal tract, RVM=rostral ventrolateral medulla.

1.7.2 Pain modulatory pathways

Wall (1967) reported increased sensitivity to noxious stimuli in decerebrate cats. The activation of pain modulatory pathways requires an input from an ascending tract or from the rostral brain. A network extends from the cortex to the periaqueductal gray, rostral ventromedial medulla, and dorsolateral pontomesencephalic tegmentum via the dorsolateral funiculus to the dorsal horn dorsolateral pontomesencephalic tegmentum. Activation of any of these areas results in antinociception (Figure 4).

The pain modulatory pathways can be activated either by electrical or chemical stimulation. Pain modulation involves various biogenic substances. Serotonin and endogenous opioids such as leu- and met-enkephalin, dynorphin, and β-endorphin are the

most common. Serotonin receptors occur in the spinal cord, and their activation produces antinociception (Crisp et al. 1991). Other studies indicate, however, that serotonin can either facilitate or inhibit spinal nociception (Eide et al. 1990). Rostral ventromedial medulla activity induces both serotonin and noradrenaline excretion and produces analgesia blocked by serotonin and noradrenaline antagonists. The effects of noradrenaline are mediated by α_2-receptors. Both dynorphin immunoreactivity and mRNA are upregulated in the spinal cord dorsal horn following inflammation induced in the rat hind paw (Millan et al. 1988, Ruda et al. 1988, Weihe et al. 1988). However, dorsal horn κ opiate-receptor immunoreactivity is unaffected by carrageenan-induced inflammation in the hindpaw, and κ opiate receptor immunoreactivity content is slightly decreased in the dorsal root ganglion and spinal dorsal horn (Ji et al. 1995). In these regions, μ-opioid receptor immunoreactivity is increased in the same situation. Similarly, μ-opioid receptor binding increases (Besse et al. 1992), and these changes together may well explain the enhanced analgesic effect of exogenous opiates after induced inflammation. Visceral sensory information is regulated in the parabrachial nucleus, where several neuropeptides occur. For example, neurotensin and somatostatin are downregulated in the parabrachial nucleus, whereas SP is first upregulated and then depleted completely after vagal stimulation (Saleh et al. 1996). These peptides play different regulatory roles in controlling visceral sensory information.

1.7.3 Pain and RFamides

NPFF was originally extracted as a FMRF-NH_2-crossreacting peptide in the bovine brain (Yang et al. 1985a); FMRF-NH_2 was established as a cardioexcitatory agent also having morphine-modulating properties (Greenberg et al. 1983, Tang et al. 1984). Yang et al. (1985b) showed that intracerebroventricular NPFF shortens tail-flick latency in naive and in morphine-treated rats. To confirm these results, antiserum against NPFF proved to be antinociceptive and to enhance morphine- and stress-induced analgesia (Kavaliers and Yang 1989). Morphine-induced analgesia is reversed by NPFF or its analogs when introduced intracerebroventricularly (Oberling et al. 1993) or into the raphe dorsalis (Dupouy and Zajac 1995). However, this morphine modulation is likely to occur indirectly, since NPFF does not bind to opioid receptors (Gouarderes et al. 1998, Dupouy and Zajac 1995). Although when injected intrathecally, NPFF potentiates morphine-induced antinociception, an effect reversed to some extent by opioid antagonists, evidence existsthat μ- and δ-opioid receptors may play a role in NPFF-produced analgesia (Gouarderes et al. 1996). It is also clear that NPFF is diminished in the spinal cord after administration of morphine or heroin (Devillers et al. 1995). Drawing together all these results, it can be stated that NPFF neurons are involved in a pain facilitatory system triggered by opioids (Devillers et al. 1995, Roumy and Zajac 1998). NPFF

analogs, produced to be stable against enzymatic degradation, are more potent than is NPFF (Gicquel et al. 1992 and 1994).

In addition to showing opioid-modulating activity, NPFF immunoreactivity is ipsilaterally upregulated in the spinal cord due to carrageenan-induced inflammation (Kontinen et al. 1997). Such inflammation reduces the potency of the descending inhibitory control of the spinal cord (Pertovaara et al. 1998). NPFF seems, however, to restore the effect of this control in a naloxone-independent manner (Pertovaara et al. 1998), providing evidence that mechanisms other than only μ-opioid reception are involved in NPFF-mediated signaling in the spinal cord. Further evidence that supraspinal controls also affect the NPFF system is provided by the work of Lombard et al. (1999). They produced prolonged monoarthritis and observed bilateral induction of NPFF binding in the spinal cord on levels 5 to 6 of the lumbar segments (L5-6), all of which was inhibited by spinal sectioning at the thoracic level.

Few studies of NPFF in neuropathic pain appeared before 1999. A study by Wei et al. (1998) investigated the effect of NPFF in the periaqueductal gray of neuropathic rats: NPFF, when introduced into the periaqueductal gray, showed antiallodynic effects in neuropathic animals. It was effective only in the monofilament-induced hindlimb withdrawal response and produced no significant alterations in the noxious heat- or noxious mechanical-stimuli tests, but in the noxious-heat response did reverse the effects of morphine.

2. Aims of the study

The purpose of this study was to:

- Clone the NPFF precursor gene in several species

Yang et al. (1985a) characterized a morphine-modulating peptide from bovine brain by using an antiserum generated against molluscan *(Macrocallista nimbosa)* FMRFamide. Two peptides were identifiable by affinity-column chromatography and reverse-phase HPLC: the NPFF and SLAAPQRF. NPFF's role in pain and other functions was thereafter extensively studied for review; see Panula et al. 1996. To further improve our understanding of the peptide and its mechanisms, it was of interest to clone the gene.

- Characterize NPFF mRNA distribution in the rat

Previous studies have shown that the NPFF peptide distribution is largely confined to the central nervous system. High expression levels exist in the spinal cord and in the pituitary. However, NPFF also has some peripheral effects (Panula et al. 1996). It was therefore of interest to characterize gene expression both in the central nervous system and the periphery. While conducting this work, another RFamide peptide, the prolactin-releasing peptide, PrRP, was characterized (Hinuma et al. 1998). Since PrRP belongs to the same family as NPFF and has some similar functions (Aarnisalo et al. 1997, Hinuma et al. 1998) it was of interest to include it for comparison and for understanding of the peptide family as a whole.

- Study RFamide gene expression during ontogeny

NPFF immunoreactivity appears in the rat embryo at embryonic day 20. The immunoreactivity thereafter spreads, reaching an adult-like appearance at the postnatal age of 4 weeks (Kivipelto et al. 1991). It was of interest to reveal the mRNA expression during embryogenesis in order to clarify the role of the peptides during ontogeny.

- Characterize essential functional properties of NPFF and PrRP

Because NPFF immunoreactivity in the spinal cord is induced in inflammatory pain (Kontinen et al. 1997), it was of interest to see whether NPFF gene expression levels would be affected in the spinal cord or in other pain-mediating areas in the CNS. The pain-modulating role of NPFF has been well studied (Panula et al. 1996), but no such data existed for PrRP, although it shares with NPFF the C-terminal RF-terminus responsible for receptor binding. It was of interest to reveal the role of PrRP in pain.

Hypophyseal NPFF originates from the hypothalamus (Majane et al. 1993) and is most probably coregulated with vasopressin (Majane and Yang 1990). PrRP is found in the hypothalamus in fibers that synapse with oxytocin-containing neuronal soma (Maruyama et al. 1999). These facts led to our investigation of NPFF- and PrRP mRNA levels in the brain during salt loading.

3. Materials and methods

3.1 Ethics

All animal experiments were carried out according to the local rules and guidelines approved by the Åbo Akademi University Animal Care and Use Committee, the Office of the Regional Governments of Western, and Southern Finland, and guidelines of the European Convention (Strasbourg, 1986).

3.2 Animals

All experiments were done on the laboratory rat, a widely used and well characterized model. Male Sprague Dawley rats were used in all experiments unless otherwise stated. Rats were housed in groups of 3 to 4, and were allowed standard laboratory pellets (Special Diet Services, Witham, Essex, UK) and fresh water *ad libitum*. The rats were kept under a 12-hour dark/light cycle.

Embryos: Sprague Dawley rats were mated overnight, with the following day counted as the first embryonic day, E1. Embryos of gestational ages E14, 15, 16, 17, 18, 19, 20, 21, and rat puppies (both male and female) postnatal day 0 (P0) were anesthetized with CO_2 and decapitated, and these embryos were quickly dissected. Sex was determined from the prepared slides under a microscope. Placentas were collected from 1 to 2 embryos of each age. Placentas and embryos were used both for immunohistochemistry and for *in situ* hybridization.

For real-time quantitative RT-PCR, embryos were kept in sterile 0.9% NaCl and dissected as quickly as possible to obtain the following organs separately: placenta, gut, kidney, adrenals, stomach, spleen, pancreas, lung, heart, thymus, spinal cord (caudal neural tube), and hindbrain (rhombencephalon), midbrain (mesencephalon), and forebrain (prosencephalon). Each type of organ from 3 to 8 individuals (all from one litter) were pooled in one tube and frozen quickly in liquid nitrogen. Samples were stored at –70°C until further analysis.

3.3 Experimental models

3.3.1 Hyperosmolar stimulus (III)

This model was designed according to earlier salt loading experiments (Sherman et al. 1986a and b, Meister et al. 1990, Majane and Yang 1991). This study used 3 groups of 6 male rats. The first group was served as a control; these animals were given tap water to drink. The second group received 2% NaCl to drink for a week. The third group received 2% NaCl for a week and then tap water for another week to see any possible rebound effect. Pellets were given *ad libitum* to all animals.

3.3.2 Methods for assessing pain ***(I)***

Rats were anesthetized with halothane. Inflammatory pain was mimicked by an injection of carrageenan (Sigma) into the left hind paw, and the effect was monitored (inflammatory signs) and by weighing of the paw of the decapitated rat.

In our work, we used the spinal nerve ligation (Kim and Chung 1992), in which the L5 and L6 spinal nerves are ligated with silk thread on the left side under pentobarbitol anesthesia. Only animals with unilateral allodynia to mechanical stimulation with monofilaments (withdrawal threshold <4.2g) were taken into the studies. For more detailed descriptions see I.

3.3.3 Injections

For the insertion of the intrathecal cannula, rats were anesthetized with a subcutaneous injection of midazolam 5.0 mg/kg (Dormicum®; Roche, Basel, Switzerland) and 1.0 ml/kg of Hypnorm® (fentanyl 0.2 mg/ml and fluanisone 10 mg/ml; Janssen Pharmaceutica, Beerse, Belgium). Intrathecal injection: A thin polyethene cannula (PE-10; Meadox Surgimed A/S, Stenløse, Denmark) was inserted through the cisterna magna into the lumbar subarachnoid space, 8 cm from the insertion, and fixed with a suture to the paravertebral muscles (Yaksh and Rudy 1976).

For intracerebral injections, the rats were implanted with a chronic guide cannula made of stainless steel (26 gauge) in a standard stereotaxic frame under general anesthesia (pentobarbitone 50 mg/kg i.p.), and behavioral testing was done as described in detail earlier (Wei et al. 2001). The injection sites were in the periaqueductal gray (PAG) (AP -1.36 mm, ML 0.7 mm, DV 6.0 mm), nucleus tractus solitarius (NTS) (AP -3.3 to -4.3 mm, ML 1.3 mm, DV 7.8 to 8.2 mm), and caudal ventrolateral medulla (CVLM) (AP -4.3 mm, ML 1.8 mm, DV 10.6 mm). After the experiments, brains were postfixed and sectioned to verify the injection sites. As the injection volume was 0.5 μl, it dispersed to an area of 0.5 mm in radius and was thus easily seen with bare eye, with no staining required.

3.4 Cloning

This dissertation project was originally aimed at cloning the NPFF gene. Rat and bovine brainstem mRNA extracts were used for rt-PCR. cDNA was synthesized from mRNA by reverse transcriptase, and a variety of oligonucleotide probes were tried for amplifying the desired sequences. The probes were designed with the knowledge that rat, bovine, and human peptides are identical (Majane et al. 1988). After successful cloning of the NPFF gene (Vilim and Ziff 1995), this study was undertaken as a collaborative effort (I).

3.5 Immunohistochemistry (I)

Tissues for immunohistochemistry were obtained from rats anesthetized with Mebunat (Orion, Helsinki, Finland) and perfused transcardially with physiological saline and a proper fixative. Our peptides were linked to keyhole limpet hemocyanin for immunizing the rabbits, and the rat tissues were fixed with 4% paraformaldehyde and then immersed in 20% sucrose overnight before freezing. Samples were kept at –20°C until sectioning on gelatine-coated (prepared in our laboratory) slides or Super Frost Plus slides (Menzel Gläser, Braunschweig, Germany). Slides were air dried for 1 to 2 hours at room temperature before storage at –20°C.

Immunohistochemistry was used to localize peptide distribution in the tissues examined. Antisera used in these studies were polyclonal rabbit antisera obtained by immunizing rabbits with intradermal injections of the peptide in question (for a detailed description see I) and then bleeding the rabbits every 10 days to obtain antiserum. The antiserum specificities were then checked by crossreactivity tests with other known peptides before being used in definitive tissue analysis.

Table 4. List of antisera used.

ANTISERUM	MADE AGAINST	DILUTION	REFERENCE	CROSS-REACTIVITY
NPFF #1E	FLFQPQRF-NH2	1:500	Kivipelto et al.1989	-
NPSF #61C	SLAAPQRF-NH2	1:1000	**I**	1 µM NPFF
PrRP20 #81B	TPDINPAWYAGRGIRPVGRF-NH2	1:2000	our unpublished observations	-
PrRP8 #86C	GIRPVGRF-NH2	1:2000	our unpublished observations	1 µM PrRP20

3.6 *In situ* hybridization (I-IV)

For *in situ* hybridization, tissues were frozen fresh in isopentane on dry ice and stored at -70°C until sectioned at -20°C. Sections were cut at -20°C to 20 µm thickness and thaw-mounted on poly-L-lysine slides (Menzel Gläser, Braunschweig, Germany).

In these works, mainly sulphur (^{35}S) radiolabeled probes were used. The protocols are described in detail in I: full-length cDNA, and in IV: oligonucleotide. In principal, RNA was first fixed in the tissues under ultraviolet light, and the tissues were then adjusted to the hybridization conditions by pipetting of prehybridization solution (similar to the hybridization solution, but with no probe) onto the slides which then remained for 1 to 2 hours at the optimal hybridization temperature. The temperature depended on the length of probe and of nucleotide content (50°C for all except PrRP oligonucleotide, for which 45°C was used). The hybridization solution contained agents that enhanced the hybridization signal and blocked

unspecific binding of the probes. The next day, excess probes were washed away under stringent conditions, and the samples were dried.

Table 5. Probes used for ISH. An equal molar mixture of a and b served for NPFF and UHR1/GPR10-like receptor *in situ* hybridizations.

PROBE AND REFERENCE	SEQUENCE	BINDS TO NUCLEOTIDES CORRESPONDING TO AMINO ACIDS
NPFF cDNA (I)	Full-length NPFF cDNA in pGEM-3Z	-
NPFF oligonucleotide a (II-IV)	5'-CAA GCA TTT CTA CCA AAC CTC TGG GGC TGA AAC AAG AAG GCT GGG TTC CTT CTA-3'	226-261 FQPQRFGRNAW
NPFF oligonucleotide b (II-IV)	5'-GGG AAG TGA TTT TGC ATG CAG ACA TAT CAC AGC AGA TGA TGT TAC TTC TT-3'	337-387 KK*HHLL*YVCMQMNHF
PrRP oligonucleotide (II-IV)	5'-TTG ATA CAG GGG TTC TTGG TCT CCA TGG AGT GCT GGT GGG CTC GGCC CTG GA-3'	64-102 SRAHQHSMETRTP
UHR1/GPR10 oligonucleotide a (II-IV)	5'-TGC AGC TCT GGG AAC CGT CGC AGA CAC ATT GCT CTC TGA AGC CTC TGC ACT-3'	105-155 SAEASESNVSATVPRAA
UHR1/GPR10 oligonucleotide b (II-IV)	5'-AGC GCC AGC ACT GCA GAT AGA GCC CAG ATG CCC AGC ACA GCG TAG GCG CTG-3'	545-595 SAYAVLGIWALSAVLA
VP CC (II)	Vasopressin exon C 241bp fragment in pGEM-3	-

3.7 Measurements and statistics

3.7.1 Measurements

In all experiments the results were obtained for at least 3 separate animals (6 in the salt-loading experiment). In analysis of embryos, each of the 3 embryos was from a different litter. Embryos collected for quantitative PCR analysis were all from the same mother; each type of organ from 3 to 8 embryos were pooled for analysis.

Samples were analyzed blindly when any two treatments (salt loading/tap water, carrageenan/vehicle) were compared. For salt loading, each of the three groups was compared with one another. For carrageenan, only one concentration of drug was used.

3.7.2 Quantification of gene expression (I, II)

Signal intensities from film autoradiographs were analyzed with a computer-assisted MCID program (Imaging Research Inc., St. Catharines, Ontario, Canada).

Individual cells and their areas were recorded under a darkfield microscope and a connected computer-assisted analysis program (analySIS) to reveal whether the treatment affected the number or size of the cells expressing NPFF or vasopressin or the PrRP gene. The areas for the whole nuclei were also measured. Grain densities were counted under a darkfield microscope by aid of a phase analysis program (analySIS) to see whether the gene expression level in an individual cell underwent any influence.

3.7.3 Statistics

Values obtained with the MCID program or analySIS were then loaded into the Prism biostatistics computer program, and paired t-tests (I), or oneway Anova followed by Bonferroni's multiple comparison tests (I-II) were performed. $P<0.05$ was considered significant.

4. Summary of results

4.1 Structure of the NPFF gene (I)

Three bioactive PQRF-peptides had been identified (Majane and Yang 1987). The gene was cloned from human, rat, bovine, and mouse tissues, and all three peptides previously found were located in the gene. All three genes have a similar organization with two introns and three exons. NPFF (FLFQRQRF-NH2) is located at the junction of exons 2 and 3; intron 2 is located in the middle of the NPFF peptide sequence. NPAF (AGEGLSSPFWSLAAPQRF-NH2) is located entirely in exon 3 of the rat. NPSF (SLAAPQRF-NH2) is located in the exon 3 of the rat (Figure 5).

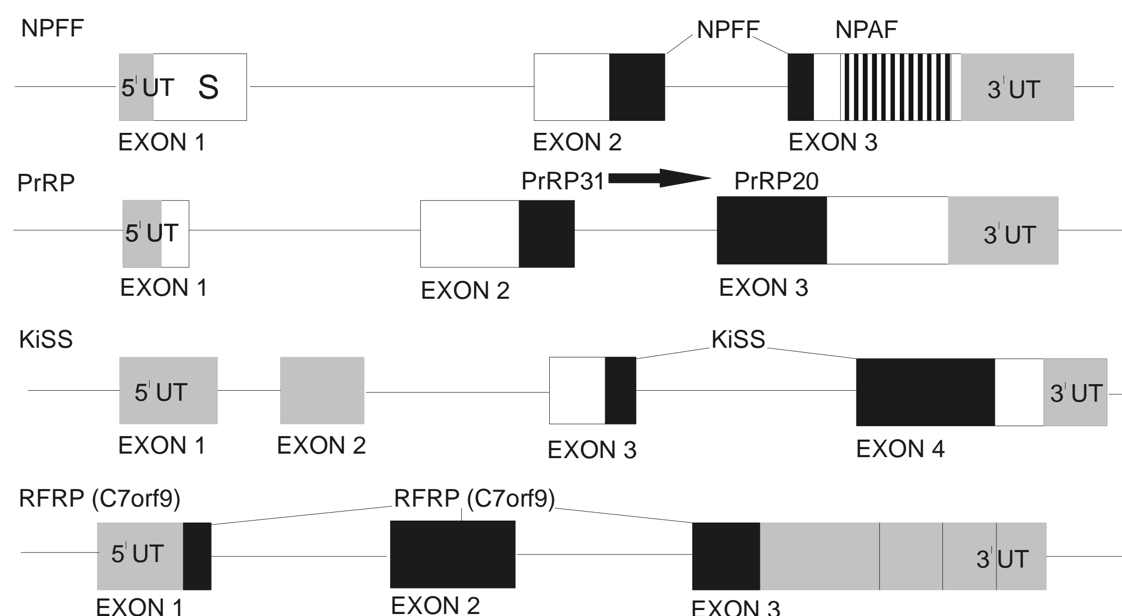

Figure 5. Comparison of known RFamide gene structures. NPFF, PrRP, and RFRP consist of three exons and two interweaving introns (lines). The KiSS precursor differs from the three others by having four exons and three introns. The light gray areas show untranslated regions, and peptide coding sequences are black or striped (NPAF). S=signal peptide. As is evident, the NPFF sequence is interrupted by the second intron in the precursor. Similarly, the PrRP31 is disrupted by an intron, but exactly at the site where PrRP20 is cleaved from PrRP31. These schemes are modified from I; (Yamada et al. 2001, West et al. 1998, Schulz et al. 2002), respectively. C7orf9 is the gene locus encoding RFRPs in the Schulz article.

The mRNAs are 600 to 800 bp in length and encode peptide precursors of 113 to 115 amino acids in length. The precursors of all species share a 40% overall identity. The genes encode C-terminal consensus sequences, and cleavage from these sites results in the bioactive peptides isolated by Majane and Yang (1987). However, the N-terminal processing site is located three amino acids N-terminally to the active amino-terminus of NPFF. Either some non-identified cleavage mechanisms exist, or the bioactive peptides undergo some degradation.

4.2 NPFF and PrRP gene expression in the rat tissues

4.2.1 Central nervous system (I-III)

According to our comparative immunohistochemical studies (unpublished observations), PrRP and NPFF/NPSF immunoreactive fibers in many areas of the CNS overlapped. PrRP immunoreactivity was characterized by an N-terminal antibody (#86C) and a C-terminal antibody (#81B), of which the C-terminal antibody gave more intensive reactions (Figures 8B vs C and E vs. F). NPFF/NPSF-like immunoreactivity was strong in the posterior pituitary (Figure 6A), whereas PrRP immunoreactivity was virtually absent from the posterior pituitary (Figures 6B and C), not demonstrating a role for PrRP in prolactin release. PrRP was ineffective when administered intrathecally (data not shown), and PrRP immunoreactivity was also absent from the spinal cord (Figure 7B), whereas NPFF/NPSF immnoreactivity was strong in the spinal cord dorsal horn laminae I and II (Figure 7A). The trigeminal nucleus displayed NPFF/NPSF immunoreactivity, but no PrRP fibers or cells were present (Figures 7C-D). PrRP immunoreactivity (data not shown) as well as PrRP mRNA expression are more prominent in the caudal nucleus of the solitary tract, but some PrRP immunoreactive fibers were also visible in the medial nucleus of the solitary tract (Figures 8A-C) where NPFF/NPSF immunoreactivity was strong (Figure 6A). Both peptides occurred in the parabrachial nucleus (Figures 8D-F), in the periaqueductal gray (Figures 9A-B), and in the thalamic paraventricular nucleus (Figures 9C-D). When the antiserum was preabsorbed with 100 µM of the peptide, all reactions were abolished (data not shown).

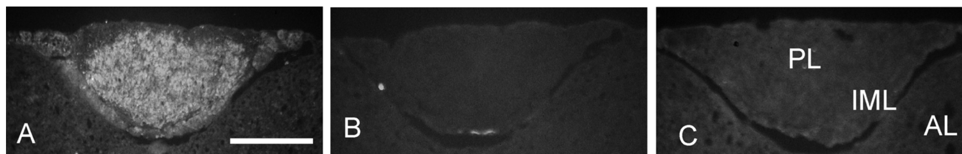

Figure 6. A: NPFF/SLAAPQRF and B: PrRP (#86) C: PrRP (#81) -immunoreactivity in the pituitary. Sections are consecutive. Pictures were taken with a fluorescent microscope with a 10x objective. Scale bar 500µm. PL=posterior lobe, IML=intermediate lobe, AL=anterior lobe.

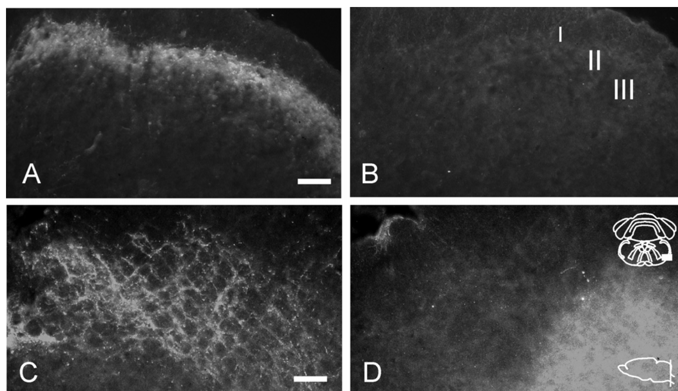

Figure 7. A: and C: NPFF/SLAAPQRF, B: and D: PrRP (#81) –immunoreactivities in consecutive sections of the spinal cord dorsal horn (A&B) and in the trigeminal nucleus (C&D). Pictures were taken with a fluorescent microscope with a 25x objective. Scale bar 500µm. I, II, and III in B mark the corresponding laminae of the spinal cord dorsal horn. The coronal level of the section of the trigeminal nucleus is visible in the lower right corner of D and the area photographed is visualized by a white rectangle on a schematized sagittal section in D.

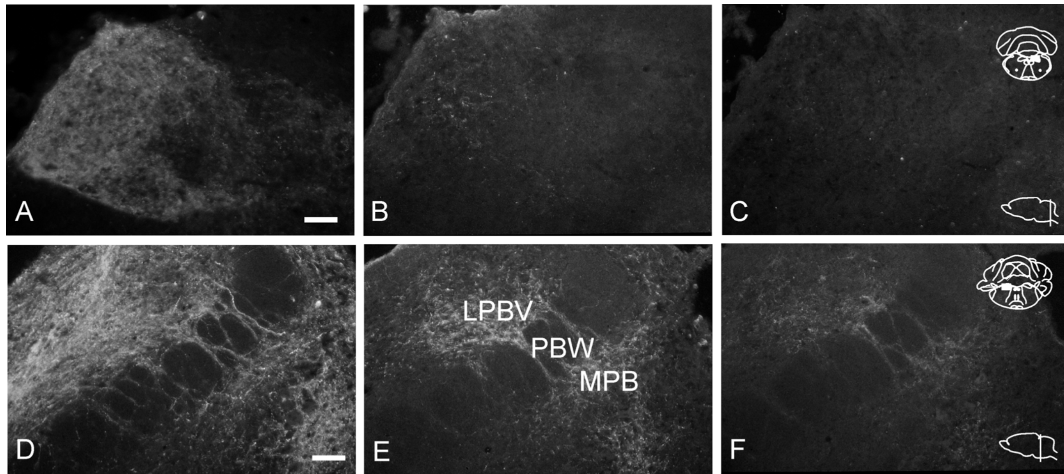

Figure 8. A: NPFF/SLAAPQRF, B: PrRP (#81) and C: PrRP (#86) immunoreactivities in consecutive sections in the medial NTS. D: NPFF/SLAAPQRF, E: PrRP (#81) and F: PrRP (#86) immunoreactivities in consecutive sections of the parabrachial nucleus. Pictures were taken with a fluorescent microscope with a 25x objective. Scale bar 500µm. The coronal level of the section of the medial NTS is visible in the lower right corner of C, and the area photographed is visualized by a white rectangle on a schematized sagittal section in C. Corresponding indications for the parabrachial nucleus are seen in F. LPBV=lateral parabrachial nucleus, PBW=waist of the parabrachial nucleus, and MPB=medial parabrachial nucleus.

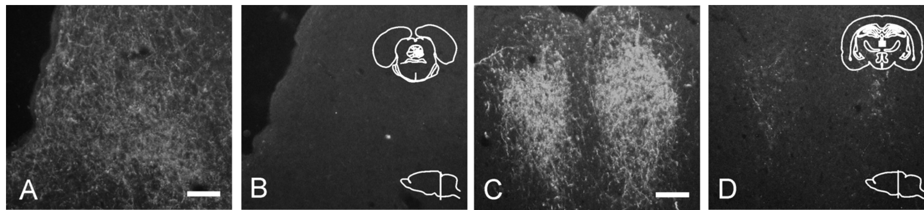

Figure 9. A: NPFF/SLAAPQRF and B: PrRP (#81) immunoreactivities in consecutive sections in the PAG. C: NPFF/SLAAPQRFand D: PrRP (#81) immunoreactivities in the thalamic paraventricular nucleus under the third ventricle in adjacent sections. Pictures were taken with a fluorescent microscope with a 25x objective. Scale bar 500µm.

In our studies (I-IV), NPFF, PrRP, and UHR1/GPR10-like receptor mRNAs were all expressed in the medulla and hypothalamus, but in different nuclei: NPFF mRNA occurred in the rostral solitary nucleus, while PrRP mRNA was expressed in the commissural part of the nucleus of the solitary tract. The UHR1/GPR10-like receptor mRNA was present in the area postrema in the medulla. NPFF mRNA was present in the hypothalamus in the paraventricular and supraoptic nuclei. PrRP mRNA was identified in an area between the dorsomedial and ventromedial hypothalamic nuclei, which is the same area where NPFF immunoreactivity previously appeared (Panula et al. 1996). Hypothalamic receptor mRNA was found in the walls of the third ventricle and additionally in the reticular thalamic nucleus. Only NPFF mRNA was found in the spinal cord dorsal horn laminae I and II. These data are summarized in a schematic drawing as Figure 10. The PrRP and UHR1/GPR10-like receptor mRNA expressions have been published by other groups (Fujii et al. 1999), and our results are in accordance with those data.

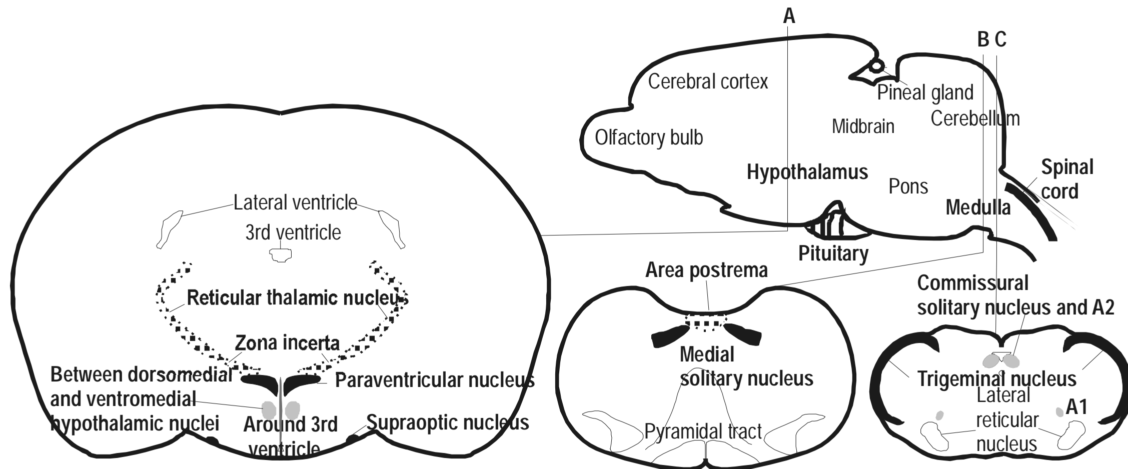

Figure 10. Schematic drawing showing NPFF (black), UHR1/GPR10-like receptor (dots) and PrRP (gray) mRNA expression in the adult rat central nervous system. Stripes=PrRP and UHR1/GPR10-like receptor simultaneously. A=Bregma -1.80—2.12 mm, B=Bregma –13.68, C= Bregma –13.80. The drawings are modified, and the coordinates are from Paxinos and Watson (1996).

4.2.2. Peripheral tissues (IV)

We observed strong expression of NPFF mRNA only in the spleen, which supports the view of a NPFF role in lymphocyte proliferation (Lecron et al. 1992). A weak, evenly distributed signal was detectable in the lung. PrRP mRNA was evident in the pancreas, adrenal medulla, testis, and epididymis. The PrRP receptor UHR1/GPR10-like receptor mRNA was found in all the same tissues as PrRP mRNA, except for the pancreas. Among the tissues examined no sign of mRNA expression of these peptides was detectable in the gut or the heart. The results are summarized in Figure 11.

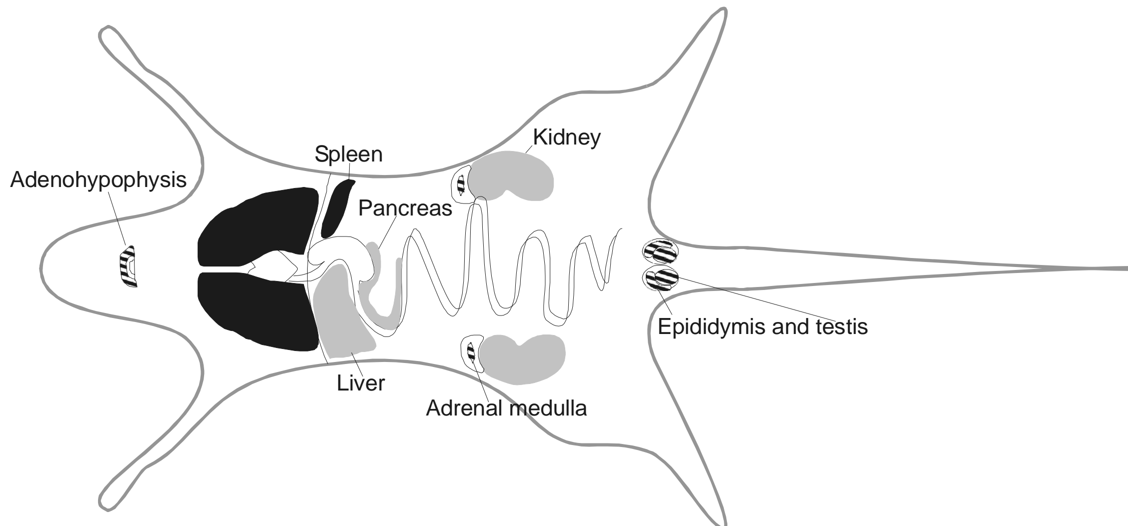

Figure 11. NPFF (black) and PrRP (gray) mRNA in peripheral tissues of the rat. Black and white stripes show areas where PrRP and UHR1/GPR10 mRNA are seen simultaneously.

4.3 Embryonal mRNA distribution of NPFF and PrRP (III)

4.3.1 Central nervous system

In this study, only NPFF mRNA was present in the youngest embryos, and appeared in the spinal cord and in the medulla. NPFF expression remained in these structures until birth and still remained in the adult rat. At E15, UHR1/GPR10-like receptor mRNA became evident in the pallidum, from whence it extended to the hippocampus-differentiating field and to the medullary reticular formation gradually at E16 and E17. NPFF mRNA appeared in the medullary reticular formation at the same time as UHR1/GPR10-like receptor mRNA. At this time, NPFF mRNA became visible also in the hypothalamus differentiating field. PrRP mRNA appeared in the CNS only at E18 in the nucleus of the solitary tract. PrRP and UHR1/GPR10 mRNA appeared in the pontine isthmus from E20 to E21. UHR1/GPR10-like receptor mRNA disappeared also from the hippocampus-differentiating field and pallidum before birth. The embryonal pituitary showed a weak but steady *in situ* hybridization signal with all three genes from E18 to P0. NPFF mRNA expression in the medullary reticular formation was transient. See Figure 12. The placenta displayed a weak but steady signal for all three genes: PrRP and NPFF mRNA were detectable in the labyrinthine part and UHR1/GPR10-like receptor mRNA in the junctional zone of the placenta.

4.3.2 Peripheral tissues

NPFF mRNA expression during development followed the same distribution as in the adult rat: NPFF mRNA occurred as a weak signal in the lung (E18-P0) and as a stronger signal in the spleen at P0. PrRP mRNA was displayed in the testis, kidney, and in the liver. UHR1/GPR10-like receptor mRNA was expressed in the adrenal gland, as in the adult rat, but was not found in the same organs as the PrRP mRNA. All three genes were expressed in the placenta throughout the ages studied. NPFF and PrRP mRNA were present in the labyrinthine part of the placenta, and UHR1/GPR10-like receptor in the junctional zone of the placenta. See Figure 12 for summary.

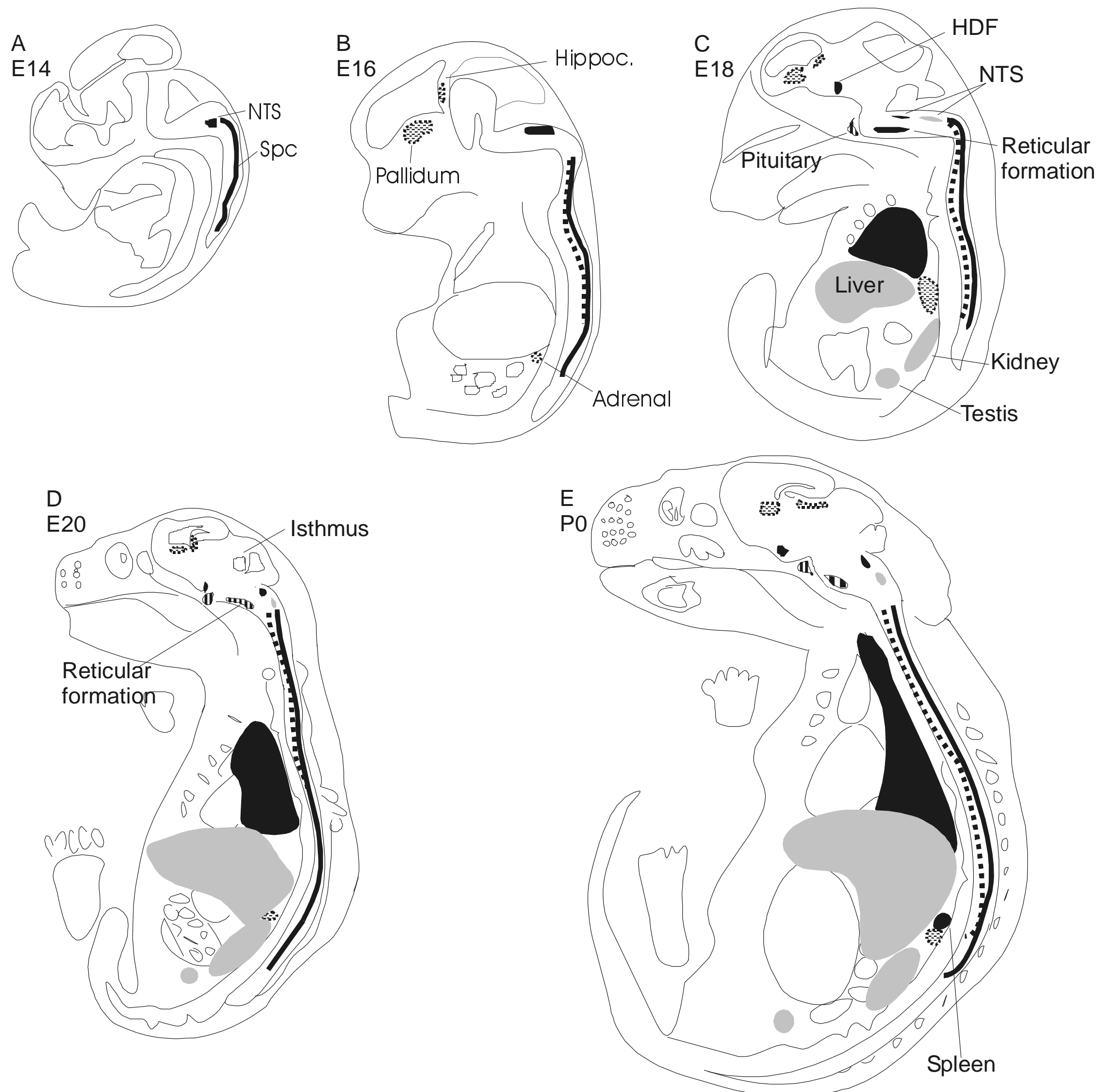

Figure 12. Schematic drawings summarizing NPFF, PrRP, and UHR1/GPR10-like receptor mRNA expression during embryogenesis in the rat. Black=NPFF mRNA, dots=UHR1/GPR10-like receptor mRNA, gray=PrRP mRNA, and black and white stripes show the areas where all three mRNAs are seen simultaneously. A: E14, B: E16, C: E18, D: E20 and E: P0. Anatomical areas are labeled when the expression is seen in them for the first time. Note that at E14 only NPFF mRNA is expressed. At E16, some areas express UHR1/GPR10-like receptor mRNA, and PrRP is first seen at E18 in the liver, testis, kidney, and NTS. The isthmus expresses PrRP and the receptor only E20-E21, and NPFF mRNA can be seen in the spleen after parturition. E=embryonal day, P=postnatal day, Hippoc=hippocampus, HDF=hypothalamus-differentiating field.

4.4. Effect of hyperosmolar stimulus on NPFF and PrRP gene expression (II)

We gave the rats hyperosmotic saline to drink for a week and after this observed that NPFF gene expression was almost completely abolished from the hypothalamic magnocellular neurons in the paraventricular and supraoptic nuclei. mRNA levels returned to initial values after a week's rehydration period (all rats had access to tap water ad libitum). NPFF mRNA in the medullary nuclei expressing the peptide gene remained, however, at control levels throughout the dehydration experiment.

PrRP is expressed in an area between the dorso- and ventromedial hypothalamic nuclei in the hypothalamus, and neither the mRNA levels of this nucleus nor medullary nuclei expressing PrRP mRNA were changed. When mRNA expression levels of the UHR1/GPR10-like receptor were studied, no apparent changes were observable in either the hypothalamic reticular nucleus or in the area postrema in the medulla.

4.5. NPFF and PrRP in pain models

4.5.1 Neuropathic pain

NPFF mRNA expression was not altered in the spinal cord after ligation of the spinal nerve ligation (I).

In our work, analgesic properties of systemically administered PrRP were studied in both normal and neuropathic rats. Intrathecal PrRP had no effect on their spinal reflexes (data not shown). $PrRP_{20}$ injected into the nucleus of the solitary tract of normal rats produced a significant (Figure 13A) dose-dependent (Figure 13B) mechanical antinociception not reversible by naloxone (Figure 13C). $PrRP_{20}$ produced only a weak hyperalgesic effect when introduced into the caudal ventrolateral medulla (Figure 13A). When $PrRP_{20}$ was injected into the nucleus of the solitary tract or central gray, it also had an antiallodynic effect (Figures 13D-E). (Unpublished observations).

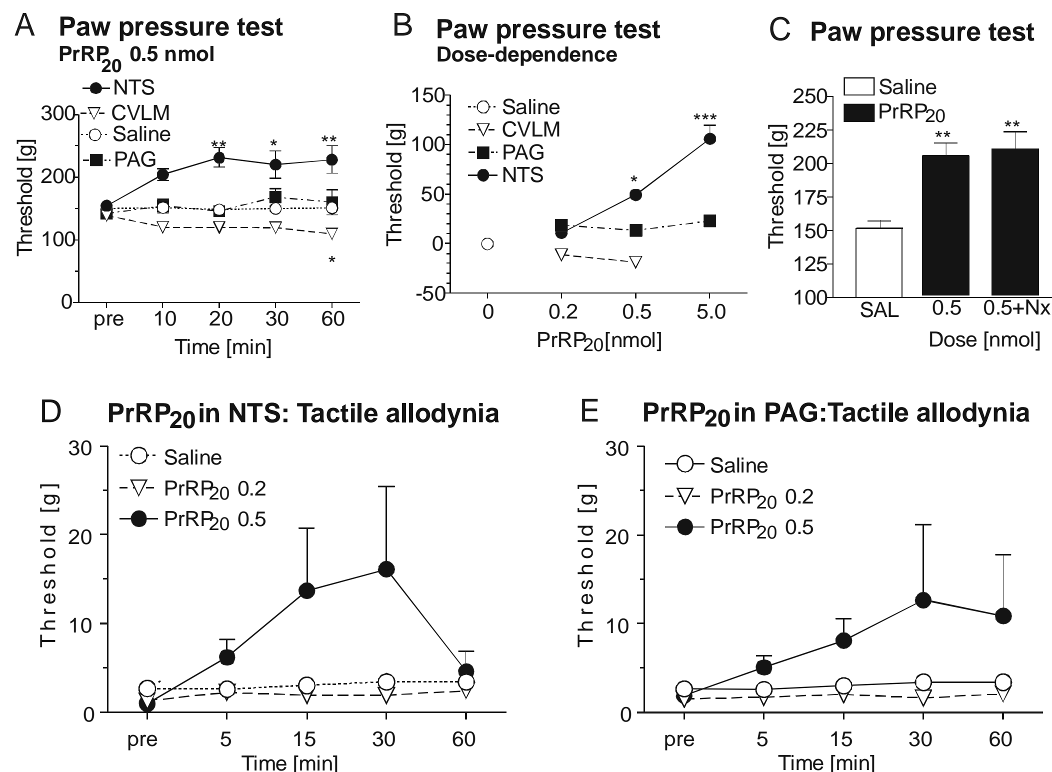

Figure 13. PrRP and pain modulation. A: The analgesic effect of PrRP is site-specific: only PrRP into the nucleus of the solitary tract (NTS) produced significant analgesia, and PrRP into the PAG had a small hyperalgesic effect. B: The analgesic effect of PrRP in the NTS was dose-dependent. C: Naloxone did not reverse the pain-modulating effect of PrRP. D: In neuropathic rats, PrRP produced a significant tactile allodynia at doses of 0.2 nmol and 0.5 nmol in the NTS, and E, in the periaqueductal gray (PAG).

4.5.2 Inflammatory pain (I)

According to our investigations, NPFF mRNA was upregulated in the spinal cord dorsal horn due to carrageenan-induced inflammation in the hind paw. Both the relative gray scale values on the autoradiographic films and the absolute number of cells expressing NPFF mRNA were increased, both by approximately 150%. These results are in accordance with changes observed in peptide levels under similar experimental conditions (Kontinen et al. 1997). PrRP or UHR1/GPR10-like receptor mRNAs are not expressed in the spinal cord (our unpublished observations), and have thus not been studied in a similar setting.

5. Discussion

5.1 RFamide peptide gene structure, expression and regulation (I)

Perry and co-workers have identified the NPFF precursor gene from human testis (Perry et al. 1997). In our work, we cloned the NPFF precursor mRNA from human, rat, mouse, and bovine brain (I). The precursor is conserved among bovine, rat, murine species and amonog human beings (Vilim and Ziff 1995, I) with overall sequence identities among all these species of 40%. In human and bovine genes, with a sequence identity of 71%, another potential peptide-ending RPamide was processed, but this was absent from the rat or murine genes. In the precursor gene, an Arg-Arg cleavage motif and Arg-Gly amidation site should produce peptides three aminoacids longer in the rat and mouse (I). According to a recent study (Bonnard et al. 2001), evidence exists of a difference in peptide maturation among animals. In the rat spinal cord, this NPA-NPFF seems to be responsible for the physiological activity of NPFF (Roumy et al. 2000), and adequate enzymes for aminoacid cleavage occur in the spinal cord. In the mouse, however, the postulated mature peptide of sequence SPA-NPFF is not detectable in the spinal cord; either the cleavage does not occur in the spinal cord but elsewhere, or the SPA-NPFF is degraded very rapidly.

Perry et al. (1997) cloned the human NPFF-related peptide by using a human genome database and PCR. They showed that the structures of the peptides deduced differ from the earlier biochemically isolated peptides. The longer peptide has two substitutions, and the shorter peptide is extended by three amino acids at its N-terminal end (human NPSF). Human NPSF requires proteolytic cleavage or other mechanisms to remove the three amino acids in order to gain a physiologically active peptide, the biochemically-isolated peptides experience a form of degradation. Alternative transcripts cannot be produced from the human NPFF gene, since it contains two introns without open reading frames or splice sites other than the ones used to produce the transcript identified from the isolated cDNA clone. They had, in accordance with our results, no alternatively spliced mRNAs detectable, despite several cloning efforts.

5.2 RFamide peptide genes in development (III)

While this study on RFamide mRNA expression during rat embryogenesis was in progress, three reports on PrRP expression during development appeared (Yano et al. 2001, Yasui et al. 2001, Reis et al. 2002). Yano et al. show that PrRP mRNA and peptide immunoreactivity are first seen in the CNS at E18 in the NTS and at E20 in the reticular nucleus of the medulla and that the signal appears only at P13 in the hypothalamus. Their results are in accordance with ours; we detected no PrRP in the hypothalamus. The latest time point assessed in our study was, however, P0. In addition to their work we reported mRNA expression in the

peripheral tissues and placenta; the placental expression was similar to that found by Reis et al. (2002) and Yasui et al. (2001).

NPFF mRNA expression differed from the appearance of NPFF immunoreactivity during embryogenesis (Kivipelto et al. 1991), and mRNA expression was evident earlier in all areas of the brain than was the peptide immunoreactivity. For example, in the spinal cord, mRNA was visible at E14, which was the earliest time-point examined, and the peptide appeared only at P3. This discrepancy could be due to a methodological problem; the antiserum used in (Kivipelto et al. 1991) is, however, still considered one of the most specific NPFF antisera available. Moreover, considering the fact that the NPFF binding sites appear postnatally between P14 and P28 (Desprat and Zajac 1994), it is reasonable that the peptide, as well, matures only postnatally. Another possibility for the differing timing of mRNA and peptide detectivity is a yet-to-be characterized mechanism of posttranslational processing in which this delay is equally long.

5.3 Functional significance of RFamide peptides

5.3.1 Body fluid homeostasis and RFamide peptides (II)

Considering the effects of PrRP in food and water intake, food intake and body weight gain were reduced, but water intake was unaffected after intracerebroventricular injection of PrRP (2-4 nmol) (Lawrence et al. 2000, and 2002). Since the catabolism induced by PrRP resembled that of leptin, they investigated the interactions of PrRP and leptin. PrRP-ir neurons in the CNS contained leptin receptors, and both had additive effects in reducing food intake and weight-gain. Both peptides reduced body temperature, a phenomenon increased when these peptides were introduced simultaneously. Sunter et al. (2001) suggest that NPFF-effected reduction in food intake is a consequence of increased water intake. In our study (II) on the effect of the hyperosmotic stimulus, PrRP mRNA levels were not significantly altered in the hypothalamus, whereas NPFF has previously and also in our study shown regulation by dehydration (Majane and Yang 1990, and 1991, Boersma et al. 1993).

PrRP is more likely to be involved in other regulatory mechanisms in the hypothalamus. A pathway of fibers reaches from the NTS to the hypothalamic neurons which synapse with the paraventricular neurons, suggesting that PrRP may play a role in control of hypothalamic hormone release (Maruyama et al. 1999). Indeed, it was thereafter shown, during the preparation of our report, that PrRP receptor-expressing neurons co-express CRH in the bed nucleus of stria terminalis (BST) (Lin et al. 2002). PrRP is therefore likely to act on BST neurons, which then modulate the PVN parvocellular neurons regulating CRH release, and in this way participate in the stress response of the hypothalamo-pituitary-axis.

In contrast to our expectations, we found, however, that the hypothalamic NPFF mRNA levels after salt loading were not augmented but drastically decreased. This decrease

is a possible result of differentially expressed transcription factors (Nystedt et al. 2002), which then may inhibit NPFF gene expression in a fashion similar to that of other sites where NPFF is not produced or may be due to a completely different mechanism. In hypothalamic stress, the morphology of the surroundings is changed, and the astrocytic processes are withdrawn from between the neurons (Hatton 1997), causing the neurons to be in closer contact with each other. These results, taken together, mean that NPFF and PrRP act through differing mechanisms for food digestion and water absorption.

5.3.2 RFamides and peripheral functions (IV)

PrRP binding sites have been characterized in various peripheral organs (Satoh et al. 2000). Indeed, we found PrRP and the UHR1/GPR10-like receptor mRNA expression to occur widely in the periphery, including the anterior pituitary, adrenal medulla, the testis, and epididymis, but in the pancreas only PrRP. We did not study adipose tissue or muscle, and in the gut investigated only the duodenum. In contrast to Satoh's work, we found no PrRP or its receptor mRNA in the heart. PrRP was originally seen as a prolactin-releasing agent, but has thereafter been shown to be involved in other mechanisms (Samson et al. 2000, Lawrence et al. 2000), and our results together with other data (Roland et al. 1999) further demonstrate the importance of PrRP as a regulator of autonomic homeostasis.

Of all peripheral tissues we examined, NPFF mRNA was expressed only in the spleen, a fact which supports NPFF's role in the proliferation of lymphocytes (Lecron et al. 1992, Minault et al. 1995). Despite the NPFF effect on gut motility (Raffa and Jacoby 1989), no NPFF immunoreactivity has been found in the gut autonomic nervous system or in the endocrine cells (Lee et al. 1993), and accordingly, we detected no NPFF mRNA in the duodenum.

5.3.3 PrRP, NPFF, and pain

The pain-modulatory effects of NPFF have been obvious since the finding of the peptide (Yang et al. 1985a). Since then, NPFF has been linked to morphine modulation (Malin 1990a and b, 1991a and b), inflammatory pain (Kontinen et al. 1997, Lombard et al. 1999), and to neuropathic pain (Courteix et al. 1999, Altier et al. 2000). Because NPFF peptide levels were upregulated in the spinal cord due to inflammation in the rat hind paw (Kontinen et al. 1997), we investigated possible changes in NPFF mRNA expression in the same area of the spinal cord after a similar inflammation in the hind paw. In accordance with the results of Kontinen et al. (1997), we found that NPFF mRNA levels were upregulated (I). Since no NPFF peptide-ir or NPFF mRNA can be found in the spinal dorsal ganglia (Panula et al. 1996), NPFF is apparently present in the local interneurons in the spinal cord laminae I and II and possibly interacts with substance P, glutamate, or dynorphin–containing interneurons (see

Figure 4). However, no morphological verification was performed, as the study aimed at cloning the NPFF gene and showing the histological distribution of NPFF mRNA.

As NPFF has morphine-modulating properties, are its effects mediated through opioid μ-, δ-, and κ-receptors (Magnuson et al. 1990,Gouarderes et al. 1996, Desprat and Zajac 1997)? Answers to this question are quite controversial, and any effects detected are small (Gouarderes et al. 1998) or indirect (Goodman et al. 1998, Ballet et al. 2002), and mostly mediated by δ-receptors (Chen et al. 2000), but NPFF evidently downregulates μ-opioid binding sites (Rothman et al. 1993, Goodman et al. 1996). It is therefore more likely that NPFF effects are mediated through other types of receptors.

Evidence indicates, however, that the antinociceptive effects of NPFF may be mediated through stimulation of nitric oxide synthase (Malin et al. 1996, Zajac et al. 2000), since intraventricular injection of 10μg NPFF produces a quasimorphine syndrome that is alleviated by 7.5 to 15 mg/kg of subcutaneous L-NAME (Malin et al. 1996).

PrRP and NPFF share a C-terminal RFamide structure, and since this structure accounts for most of the binding of the protein to its receptor, we assessed the involvement of PrRP in pain. Lin et al. (2002) also propose that PrRP may play a role in nociception, as UHR1/GPR10-like receptor is co-expressed with enkephalin in the parabrachial nucleus. The pain-modulating effects of PrRP were clear in our study, but obviously the mechanisms differ from those of NPFF, as can be seen from Table 6.

Table 6. Pain modulation by NPFF and PrRP in neuropathic (Ne) and normal animals (ctrl).

SITE OF ADMINISTRATION	**PEPTIDE/DOSE**	**EFFECT**	**RECEPTORS INVOLVED**	**REFERENCES**
Ventricle	NPFF/dose -> *PrRP*	Antiallodynic (15 nmol) in ne, hyperesthetic (0.8 nmol) and analgesic in ctrl *Not studied*	Partly μ-opioid receptor	Altier et al. 2000 Oberling et al. 1993
Periaqueductal gray	NPFF/0.05 nmol *PrRP/0.5 nmol*	Antiallodynic in ne, *Analgesic in ne, no effect in ctrl*	Partly μ-opioid receptor *Not known*	Wei et al. 1998 *our unpublished observations*
Nucleus of the solitary tract	NPFF *PrRP/0.5 nmol*	Not studied *Analgesic in both*	*Not μ-opioid receptor*	*our unpublished observations*
Caudal ventrolateral medulla	NPFF *PrRP/0.5 nmol*	Not known *Hyperalgesic in ctrl*	*Not known*	*our unpublished observations*
Spinal cord	NPFF/5 nmol *PrRP/0.5-10 nmol*	Analgesic in both *No effect*	Partly μ-opioid receptor	Xu et al. 1999 *our unpublished observations*

The doses used for PrRP were pharmacological, and the amount of endogenous neuropeptide released in a single synaptic cleft may also be large although the neuropeptide concentration in a given amount of tissue is often low. As the injection volume was 0.5 μl,

and dispersed to a larger area than a single nucleus, the effects of PrRP may be mediated through any adjacent area to the site of administration.

5.4 Expression of the UHR1/GPR10 -like receptor

The UHR1/GPR10-like receptor mRNA is expressed in the reticular thalamic nucleus and zona incerta, in the periventricular area of the 3rd ventricle, and in the area postrema in the medulla (Roland et al. 1999, Ibata et al. 2000, II-IV), and in the paraventricular nucleus, amygdala, and bed nucleus of the stria terminalis (Lin et al. 2002). The peripheral tissues expressing UHR1/GPR10-like receptor are anterior pituitary, adrenal medulla, and male reproductive organs (IV, V). Lin et al. used different primers for obtaining the cDNA probes than did Roland et al., and we have used synthetic oligonucleotides. Because *in situ* hybridization is a technically demanding method, it is possible that areas of lower expression are not always visible.

As we have shown that PrRP (10 nmol-1 μmol) and NPFF (1-10 nmol *in vitro*) were both involved in at least prolactin release and pain modulation and that they had a common RFamide structure, it is not surprising that recently Engström et al. (2003) discovered the binding of PrRP to NPFF2 receptors.

5.5 Methodological considerations

Several methods exist for *in situ* hybridization: fluorescence, digoxigenin, or radiolabeling with ^{125}I, ^{33}P, or ^{35}S. Probes can be synthetic or full-length or fragments of cDNA grown in plasmids. The choice was easy, since the radioactive method using ^{35}S was already established in our laboratory, and the gene expression of NPFF was so low that it was difficult to detect with digoxigenin-labeled full-length cDNA (data not shown). The advantage of radiolabeling the probes rather than immunolabeling is the longivity of the data, but also in addition to slides, the possibility to analyze samples from autoradiographs. The choice between radioligands was determined by safety: the radiation diameter of ^{35}S is shorter than that of ^{125}I or ^{33}P, but the signal strong enough. The advantage of using synthetic oligonucleotides rather than cDNA is the simplicity of the procedure, its good reproducibility, and avoidance of many toxic agents.

We used fluorescent immunohistochemistry. The avidin-biotin-complex is another broadly used detection system, and is based on the high affinity of avidin to biotin. The advantage of fluorescent labeling is its speed and simplicity. Avidin-biotin-complex staining is performed on free-floating tissue sections, which permits the use of more diluted antisera (e.g., 1:10 000 vs. 1:2 000). This method's multiple steps and harmful chemicals are its greatest disadvantages. On the other hand, the samples remain forever, unlike the fluorescent dye that slowly fades away in weeks.

Our antibodies were tested for possible cross-reactivities before their use in experimental settings (for details, see Kivipelto et al. 1989). In the early 1980's, FMRFamide stainings were confusing, since the antibodies crossreacted with NPY which shares a similar C-terminal structure with FMRFamides (Hökfelt et al. 1983). The antibodies used these studies crossreacted neither (I) with NPY nor with related peptides.

Inflammatory pain can be mimicked by introducing either formalin (Dubuisson and Dennis 1977), carrageenan (Kayser and Guilbaud 1987,Hylden et al. 1991), or complete Freund´s adjuvant (mineral oil containing killed *Mycobacterium butyricum* at 10mg/ml) into the skin (Iadarola et al. 1988) or a joint (Schaible et al. 1987) of an animal. The inflammatory response lasts from 1 to 8 hours. The response is limited to the injection site, with no signs of systemic disease (Iadarola et al. 1988) or other side-effects. Carrageenan is widely used in our laboratory and was therefore chosen for these studies.

Neuropathic pain is caused by injury to the nerve followed by plastic changes in neuronal circuitry and pain perception. Experimental neuropathic pain can be produced by ligating the sciatic (Bennett and Xie 1988, Seltzer et al. 1990) or spinal nerves (Kim and Chung 1992) of experimental animals. Assessment involves testing with mechanical and thermal stimulation for the effect of the neuropathic surgery. The advantage of spinal nerve ligation is the standard surgical procedure and the fact that injured nerves are completely separated from intact nerves.

6. Summary and conclusions

- The NPFF precursor gene was cloned (I) and found to be identical with a sequence identified earlier (Perry et al. 1997). The precursor mRNA contained the sequences for all three biochemically active peptides, NPFF, NPAF, and NPSF, and it was preserved among the species tested: rat, mouse, bovine, and human.

- The distribution of NPFF mRNA was characterized throughout the rat tissues. This peptide was prominently expressed in the central nervous system: hypothalamic paraventricular and supraoptic nuclei, medullary nucleus of the solitary tract and trigeminal complex, and the spinal cord dorsal horn (I). Of the peripheral tissues, NPFF mRNA was found only in the spleen (IV). The embryonal distribution corresponded largely to the adult NPFF mRNA-expressing areas but differed from the NPFF immunoreactivity found in the embryo (III). Thus, there is a considerable delay in translating the gene into a mature protein, and this may be due to a post-translational regulatory mechanism yet to be characterized.

- NPFF immunoreactivity has been found in the hypothalamus in an area (Kivipelto et al. 1989) with no NPFF mRNA present (I), which led to the question of other RFamide peptides. Hinuma *et al.* (1998) cloned PrRP, and PrRP mRNA was indeed found exactly in the hypothalamic nucleus exactly where NPFF immunoreactivity but not mRNA was evident. Among other researchers, we characterized PrRP mRNA distribution in the brain and periphery (IV). PrRP was expressed in the male reproductive organs both in embryo and in adult rats, and in the kidney and liver (III, IV). The PrRP and UHR1/GPR10-like receptor mRNA expression during embryogenesis largely corresponded to the adult distribution (III), except for the hypothalamic nuclei, which began to express PrRP only postnatally.

- NPFF mRNA expression was enhanced in response to the carrageenan inflammation in the spinal cord ipsilaterally to the inflammation (I). PrRP was also involved in nociceptive pathways, not via the spinal cord but through the medulla. PrRP (0.5 nmol) was analgesic when injected into the nucleus of the solitary tract (dorsal medulla) in normal and in neuropathic rats and analgesic also when injected into the periaqueductal gray of neuropathic but not of normal rats. The PrRP-mediated analgesia was not mediated via μ-opioid receptors.

- Contrary to our expectations, during chronic hyperosmolar stress the hypothalamic NPFF mRNA was not up- but downregulated (II), neither medullary NPFF, PrRP, nor UHR1/GPR10-like receptor mRNA was affected by the treatment. No significant changes were evident in hypothalamic PrRP or UHR1/GPR10-like receptor mRNA levels. This result suggests a possibly novel transcriptional regulatory mechanism.

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