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NOCICEPTIN/ORPHANIN FQ: THE NOVEL PEPTIDE WITH MULTIPLE OLD REGULATORY FUNCTIONS

Mila Vlaskovska and Lyubomir Kasakov
Department of Pharmacology, Medical University, Sofia, Bulgaria and Institute of Physiology, Bulgarian Academy of Sciences, Sofia, Bulgaria

The identification some years ago of the opioid-like heptadecapeptide nociceptin/orphanin FQ (NOFQ) as the endogenous ligand of orphan opioid like-1 (ORL1) receptor evoked a rapidly growing interest in understanding the physiological significance of this peptide. This initiated a great number of investigations aimed at studying the cellular and integrative effects of NOFQ and disclosing the mechanism of its action. The number of publications in this field is increasing constantly and a great deal of information along with some controversial data is being already accumulated. In the present article we attempted to summarise most of the current information about NOFQ generation, metabolism, mechanisms of action and the cellular and integrative effects and to emphasise the possibilities for clinical implications. ***Biomed Rev 2000; 11: 1-17.***

ORPHAN RECEPTOR

The classical opioid receptors belong to 3 distinct subclasses which are μ-, κ- and δ-type (reviewed in 20,91). An atypical subclass called "orphan" receptor, for which no specific endogenous ligand had been identified, was associated to the opiate receptor superfamily. In 1992 the gene encoding δ-opioid receptor (DOR) was cloned (27,53) which was followed by the cloning of two other genes that encode κ- (116) and μ- (14) opioid receptors, KOR and MOR, respectively. A fourth distinct gene was partially characterised and found to encode the sequence of the orphan receptor (reviewed in 68).

Nomenclature

Several laboratories reported for isolation of complementary DNA (cDNA) encoding the amino acid sequence of NOFQ receptor in various species that was referred to by several names: a human clone called ORL1 (74), a rat clone termed differently as C-3 (57), FLAT7-5EU (111), Hyp8-1 (112), LC132 (6), oOR (38), oprl (15), ROR-C (31), XOR1 (109) and a mouse clone called MOR-C (83). The mouse clone termed KOR3 has later been identified as κ3-opioid receptor (88). Further in this review NOFQ receptor and ORL1 receptor will be used interchangeably.

Gene and molecular organisation

The gene of the ORL1 receptor was mapped and located to the distal region of chromosome 2 in mouse (15), or to region q13.2-13.3 of chromosome 20 in man (52). The protein coding sequence of mouse ORL1 gene was interspaced by one intron located within the codon for arginine residue of first cytoplasmic loop and a second intron within the codon of glutamic acid residue of second exofacial loop. The same intron was detected as a stretch in 118 bp of human gene (75).

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Correspondence and reprint requests to Dr Mila Vlaskovska, Department of Phamacology, Medical University, BG-1431 Sofia, Bulgaria. E-mail: vlaskov@medfac.acad.bg

Identical organisation is verified in the opioid receptors which strongly implies that all four genes have evolved from a common ancestor and belong to one superfamily class. The ORL1 receptor is G protein-coupled which cDNA has been cloned. Murine ORL1 receptor consists of 367 residues being shorter by 3 residue insertions in N-terminal domain than human receptor. cDNA deduced amino acid sequences highlighted a considerable homology of opioid and ORL1 receptors. The data are summarised in Table 1. The second exofacial loop resembles more closely κ- than μ- or δ-opioid receptors (Fig. 1). Biochemical evidence has recently been found by studying the inhibition of ^{125}I-Tyr^{14}-NOFQ binding of various opioids and NOFQ peptides or by inhibition of forskolin-stimulated cAMP accumulation which suggests that NOFQ receptors could represent a heterogeneous family which is responsible for the diverse behavioral effects of NOFQ (65).

Recent evidence shows that ORL1 receptor-mediated pertussis toxin-sensitive signals and stimulation of a mitogen-activated protein (MAP) kinase, suggesting that these receptors are capable of coupling to Gi/Go proteins. The same effect is mediated via μ-opioid receptors (41).

Table 1. *Sequence homology between ORL1, μ-, δ-, and κ-opioid receptors*

HOMOLOGY		
HIGH	MEDIUM	LOW
Segments		
1. *Segment 2:* 77.3% (11/22* residues)	*Segment 5:* 56.5% (13/23 residues)	*Segment 4:* 21.7% (5/23 residues)
2. *Segment 7:* 75.0% (15/25 residues)	*Segment 1:* 50.0% (11/24 residues)	
3. *Segment 3:* 72.7% (16/22 residues)	*Segment 6:* 40.9% (9/22 residues)	
Loops		
1. *Loop 1:* 85.4% (6/7 residues)		
2. *Loop 3:* 75.0% (18/24 residues)		
3. *Loop 4:* 75.0% (9/12 residues)		
4. *Loop 2:* 59.1% (13/22 residues)		

* identical/total

Anatomical distribution

In numerous investigations (6,31,56a,74,111) the strong presence of ORL1 transcripts was located by histochemical hybridisation in: (*i*) cortical and limbic areas (amygdala, hippocampus, habenula, septum), (*ii*) hypothalamus (paraventricular and ventromedial nuclei), (*iii*) sympathetic neurons, (*iv*) brain stem (locus coeruleus, central grey matter, dorsal raphe and parabrachial nuclei), and (*v*) ventral and dorsal horn of the spinal cord and dorsal root ganglion. ORL1 immunohistochemical visualisation by antibody to the receptor N-terminal revealed identical distribution which suggested that ORL1 receptor is located on neurons placed in the local circuits (3) contrasting the localised presence of prepro-NOFQ mRNA predominantly on interneurons (46). It is plausible to suggest that NOFQ released from interneurons should suppress the neighboured neurons. However, no detectable expression of ORL1 receptor gene was found in the striatum and cerebellum. The specific localisation of ORL1 receptor in discrete brain areas (99) strongly implies that ORL1 receptors are most likely involved in the modulation of a variety of central nervous ststem (CNS) processes including memory and learning, emotions and attention, movements and tone,

Abbreviations used

Amino acids: A, Alanine (Ala); C, Cysteine (Cys); D, Aspartic acid (Asp); E, Glutamic acid (Glu); F, Phenylalanine (Phe); G, Glycine (Gly); H, Histidine (His); Ile, Isoleucine (Ile); K, Lysine (Lys); L, Leucine (Leu) M, Methionine (Met); N, Asparagine (Asn); P, Proline (Pro); Q, Glutamine(Gln); R, Arginine (Arg); S, Serine (Ser); T, Threonine (Thr); V, Valine (Val); W, Triptophan (Trp); Y, Tyrosine (Tyr); BPT, Bacillus pertussis toxin; CCK, cholecystokinin; CGRP, calcitonin gene-related peptide; CRF, corticotropin releasing factor; DA, dopamine; DG, hippocampal dentate gyrus; DyA, dynorphin A; (m)EPSC, (miniature) excitatory postsynaptic current; ESI MS, electrospray ionisation mass spectrometry; FPLC, fast protein liquid chromatography; GABA, gamma amino butyric acid; GIRK channel, G-protein-activated inwardly rectifying potassium channel; HPLC, high performance liquid chromatography; icv, intracerebroventricular; (m) IPSC, (miniature) postsynaptic current; ith, intrathecal; LTP, long-term potentiation; N and numbers following, NOFQ fragment; NC(1-13), [Phe^{1}Y(CH_2-NH)Gly^2]NC(1-13)NH_2; NO, nitric oxide; NOS, nitric oxide synthase; NPY, neuropeptide Y; NST, nocistatin; ORL1, orphan opioid like-1 receptor; PAG, periaqueductal grey matter; PG, prostaglandin; pIC, negative logarithm of agonist concentration which produces 50 % of the maximal inhibitory effect; PNP- prepronociceptin peptide; RIC, reconstructed ion chromatogram; SIA, stress-induced analgesia; SNP, sodium nitroprusside; SP, substance P; TTX, tetrodotoxin; VGCC, voltage gated calcium channel; VIP, vasoactive intestinal polypeptide; VTA, ventral tegmental area.

Figure 1. *Amino acid sequence of human ORL1, δ (DOR), κ (KOR), and μ (MOR) opioid receptors*

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Line 1: 1    1          10         20         30         40         50
Line 2: 60   60         70         80         90         100        110
Line 3: 120  120        130        140        150        160        170
Line 4: 180  180        190        200        210        220        230
Line 5: 240  240        250        260        270        280        290
Line 6: 300  300        310        320        330        340        350
Line 7: 360  360        370        380        390        400        410
Line 8: 420  420        430        440        450        460        470
Line 9: 480  480        490        500
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ORL1                                    MEPLFPAPFWEVIYGSHLQGNLS 1
                LLSPNHSLLPPHLLLNASHGAFLPLGLKVTIVGLYLAVCVGGLLGNGLVM 2
                YVILRHTKMKTATNIYIFNLALADTLVLLTLPFQGTDILLGFWPFGNALC 3
                KTVIAIDYYNMFTSTFTLTAMSVDRYVAICHPIRALDVRTSSKAQAVNVA 4
                IWALASVVGVPVAIMGSAQVEDEE..IECLVEIPTPQD..YWGPVFAICI 5
                FLFSFIVPVLVISVCYSLMIRRLRGVRLLSGSREKDRNLRRITRLVLVVV 6
                AVFVGCWTPVQVFVLAQGLG.VQPSSETAVAILRFCTALGYVNSCLNPIL 7
                YAFLDENFKACFRKFCCASALRRDVQVSDRVRSIAKDVALACKTSETVPR 8
                PA
δ-opioid                                      MEPAPSAGAELQPPLFANASD 1
                AYPSAFPSAGANASGPPGPGSASSLALAIAITALYSAVCAVGLLGNVLVM 2
                FGIVRYTKMKTATNIYIFNLALADALATSTLPFQSAKYLMETWPFGELLC 3
                KAVLSIDYYNMFTSIFTLTMMSVDRYIAVCHPVKALDFRTPAKAKLINIC 4
                IWVLASGVGVPIMVMAVGRPRDGA..VVCMLQFPSP..SWYWDTVTKICV 5
                FLFAFVVPILIITVCYGLMLLRLRSVRLLSGSKEKDRSLRRITRMVLVVV 6
                GAFVVCWAPIHIFVIVWTLVDIDRRDPLVVAALHLCIALGYANSSLNPVL 7
                YAFLDENFKRCFRQLCRKPCGRPDPSSFSRPREATARERVTACTPSDGPG 8
                GGRAA                                               9
κ-opioid        MDSPIQIFRGEPGPTCAPSACLPPNSSAWFP                     1
                GWAEPDSNGSAGSEDAQLEPAHISPAIPVIITAVYSVVFVVGLVGNSLVM 2
                FVIIRYTKKKTATNIYIFNIALADALVTTTMPFQSTVYLMNSWPFGDVLC 3
                KIVISIDYYNMFTSIFTLCTMMSVRYIAVCHPVKALDFRTPLKAKIINIC 4
                IWLLSSSVGISAIVLGGTKVREDVDVIECSLQFPDDDYSW.WDLFMKICV 5
                FIFAFVIPVLIIIVCYTLMILRLKSVRLLSGSREKDRNLRRITRLVLVVV 6
                AVFVVCWTPIHIFILVEALGSTSHSTA.ALSSYYFCIALGYTNSSLNPIL 7
                YAFLDENFKRCFRDFCFPLKMRMERQSTSRVRNTVQDPAYLRDIDGMNKP 8
                V                                                   9
μ-opioid                MDSSAAPTNASNCTDALAYSSCSPAPSPGSWVNLSNLDGNLS 1
                DPGGPNRTLNGGRDSLCPPTGSPSMITAITIMALYSIVCVVGLFGNFLVM 2
                YVIVRYTKMKTATNIYIFNIALADALATSTLPFQSVNYLMGTWPFGTILC 3
                KIVISIDYYNMFTSIFTLCTMSVDRYIAVCHPVKALDFRTPRNAKIINVC 4
                NWILSSAIGLPVMFMATTKYRQGS..IDCTLTFSHP..TWYWENLVKICV 5
                FIFAFIMPVLIITVCYGLMILRLKSVRMLSGSKEKDRNLRRITRMVLVVV 6
                AVFIVCWTPIHIYVIIKALVTI.PETTFQTVSWHFCIALCYTNSCLNPVI 7
                YAFLDENFKRCFREFCIPTSSNIEQQNSTRIRQNTRDHPSTANTVDTRTN 8
                QLENLEAETAPLP                                       9
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Transmembrane domains: TM1 (81-104), TM2 (112-133), TM3 (151-173), TM4 (196-218), TM5 (248-270), TM6 (295-316), TM7 (335-354)
Intracellular loops: IL1 (105-111), IL2 (174-195), IL3 (271-294)
Extracellular loops: EC1 (134-151), EC2 (219-247), EC3 (317-334)

neuroendocrine functions and overall homeostasis as well as all kinds of sensory perception (nociceptive, gustatory, olfactory, auditory and visual). Strong ORL1 receptor mRNA signal was identified in the intestine, vas deferens, liver and spleen but not in the skeletal muscle, kidney, testis and adrenal gland (109). Lymphocytes were found to express a strong $mRNA^{ORL1}$ signal (112) that suggests the participation of ORL1 receptor in the immune reactions. The embryonic brain shows a strong and dense expression of prepro-NOFQ- and ORL1 transcripts in the whole mantle zone (46). An intriguing finding is the colocalisation and overlapping of ORL1 receptor mRNA with GIRK1 (G-protein-activated K^+ channel) mRNA revealed by *in situ* hybridisation using oligonucleotide probes (45). The microscopic evidence strongly suggests that both are colocalised in the same neuron.

Binding characteristics

The molecular organisation of ORL1 receptor highlighted a strong structural homology with the classical opioid receptors. One had eventually to anticipate a similar binding of opioids to ORL1 receptor. However the binding properties of ORL1 receptor appear to be quite dissimilar to opioid receptors. The recognition (Ki) and activation (ED50) parameters of ORL1 receptor estimated in radioligand experiments demonstrated that morphine, naloxone, ethylketocyclazocine, diprenorphine, nor-binaltrophine, DAMGO (D-Ala^2, N-Met-Phe^4, Gly-ol^5-enkephalin), DTLET (D-Thr^2, Leu^5, Thr^6-enkephalin), Met-enkephalin (Methionine^5-enkephalin), Leu-enkephalin (Leucine^5-enkephalin), α-neoendorphine, β-endorphine, U-50488 were inactive (did not bind) at these receptors (Ki and ED50 > 10 μM against 0.13 and 0.8 nM, respectively for NOFQ) (7). Lofentanil (Ki 24 nM) and dynorphin A (Ki 110 nM) were reported the only substances which bind to ORL1 receptor. This is an indication that ORL1 receptor might contain some functional site(s) that has an insufficient capability of binding opioids. However, mutation of 4 residues Ala216, Val279, Gln280 and Val281 in ORL1 receptor sequence to correspondingly located residues Lys227, Ile290, His291 and Ile292 in KOR sequence enabled the mutant ORL1 receptor of binding with high affinity bremazocine, a non-type selective opioid ligand (98). Precise saturation and displacement receptor binding studies have revealed single high-affinity (Kd 21.6-116.7 pM) and high-capacity binding site for NOFQ (2). The authors calculated that one receptor site possesses the capacity of stimulating 10 G-protein binding sites.

NOCICEPTIN/ORPHANIN FQ

In 1995 two research groups have independently reported for the isolation, amino acid sequencing and pharmacological characterisation of a novel brain heptadecapeptide that binds specifically to the ORL1 receptor. The results have been published on 12 October 1995 in *Nature* (69) and on 3 November 1995 in *Science* (90). Meunier's group (69) purified in three consecutive steps (size-exclusion chromatography, cation-exchange FPLC and reversed-phase HPLC) an acidified extract of rat brains and determined the molecular mass and amino acid sequence of the active component (bioassayed on CHO/ORL1 receptor-bearing cell line for inhibition of forskolin-induced intracellular accumulation of cAMP) by mass spectrometry. The authors found that injected intracerebroventricularly (icv) in mice the peptide induces hyperalgesia, hence termed it nociceptin. Reinscheid's group (90) purified the acetic extract of porcine hypothalamus by cation-exchange HPLC with further fractionation of the active component (bioassayed similarly on LC132/ORL1 receptor-transfected cells) through 5 reversed-phase HPLC steps. The material has been analysed by mass spectrometry and sequenced by means of Edman degradation. The authors found that in mice icv application of the peptide lowered the locomotor activity and produced hyperalgesia in tail-flick test, and termed it orphanin FQ (F and Q being single letter code of N- and C-terminal amino acid) due to its affinity to ORL1 receptor.

Identification

NOFQ molecule consists of 17 amino acid residues with F positioned at the end of N-terminal and Q positioned at the end of C-terminal (Fig. 2). It is representing a strong similarity to other short chain opioid peptides among which dynorphin A (DyA) is most closely related.

Figure 2. *Amino acid sequence of NOFQ and related opioid peptides*

Peptide	**Amino acid residues and position***																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
NOFQ	F	**G**	**G**	**F**	T	G	A	R	**K**	**S**	A	R	**K**	**L**	A	**N**	**Q**
α-Endorphin	Y	**G**	**G**	**F**	M	T	S	E	**K**	**S**	Q	T	P	**L**	V	T	
Dynorphin A	Y	**G**	**G**	**F**	L	R	R	I	**R**	P	K	L	**K**	W	D	**N**	**Q**
χ-Endorphin	Y	**G**	**G**	**F**	M	T	S	E	**K**	**S**	Q	T	P	**L**	V	T	L
Dynorphin B	Y	**G**	**G**	**F**	L	R	R	Q	F	K	V	V	T				
Leu-Enkephalin	Y	**G**	**G**	**F**	L												
Met-Enkephalin	Y	**G**	**G**	**F**	M												
Endomorphine 1	Y	P	W	**F**													
Endomorphine 2	Y	P	F	**F**													

**The amino acids homologous for NOFQ and other peptides are shown with bold letters.*

The N-terminal fragment is reminiscent of YGGF that is the N-terminal quadruplet of dynorphins, endorphins and enkephalins. NOFQ bound with a high affinity to ORL1 receptor and synthetic peptide was identical to natural peptide in its pharmacological activities. A striking difference with all other peptides is the replacement of tyrosine by phenylalanine in NOFQ molecule.

Precursor

NOFQ molecule is a portion of precursor polypeptide termed prepronociceptin (preproNOFQ). The gene for the precursor is located to the short arm of human chromosome 8 (8p21) (75). The gene consists of at least 4 exons with a structural organisation resembling mostly the genes encoding precursors of endogenous opioid peptides. It encodes a single copy of NOFQ contrasting the preprodynorphin or preproenkephalin genes which encode more than one copy of L- and M-enkephalin. The localisation of preproNOFQ gene suggests that the role of gene in the pathogenesis of human disease is unlikely (75). NOFQ sequence is flanked by dibasic amino acid motifs (Fig. 3) in the rather conserved precursor molecule (69).

Localisation

The localisation of NOFQ immunoreactive fibres in the brain is limited to a few particular areas contrasting the wider distribution of ORL1 receptor. Northern blot studies have shown that preproNOFQ gene is expressed in brain and spinal cord of human and rat as a 1.3 kb long mRNA (75,85). The highest level of expression were found in amygdala, intermediate hypothalamus, thalamus, and substantia nigra. Lower levels were described in hippocampus and corpus callosum. The 1.3 kb mRNA was described in the rat ovary, human spleen, leucocytes and fetal kidney. Interestingly, preproNOFQ mRNA was absent in the adult brain (85). Precise studies in which *in situ* hybridisation and immunocytochemistry have been employed, revealed a dense preproNOFQ gene distribution in the central gray matter tegmental field, nucleus of lateral lemniscus, superior olive and the trigeminal nuceus of the brain stem. Lower density was described in the cortical layers I, II and III, lateral septum, nucleus of lateral olfactory tract, bed nucleus of stria terminalis, lateral geniculate nuscleus, medial preoptic, ventromedial and supramammilary hypothalamic nuclei, pontine nuclei, inferior colliculus, nuclei of the raphe and lateral lemniscus. Sparse distribution was described in olfactory bulb and hippocampal CA1 region. In the spinal cord the strongest preproNOFQ gene signal has been found in laminae III-I of dorsal horn and lamina X. In contrast, mRNA transcripts has not been detected in caudate-putamen and cerebellum (44,97). Riedl *et al* (92) have reported for a high amount of NOFQ in superficial dorsal horn, lateral spinal nucleus and dorsal zone to central canal. The authors presented convincing evidence that NOFQ positive processes survived unilateral rhizotomy which implies a central rather than a peripheral origin of the processes. Despite the presence of NOFQ in areas related to pain perception, double stained colour microscopic analysis showed that opioid peptides and NOFQ did not usually coexist. On the contrary, NOFQ and opioid peptide precursors are localised separately in different terminals from which can be separately released thereby modulating the pain in an opposite way (97). In an overall scheme of the localisation it might be true that NOFQ distribution matches well the presence of ORL1 receptor.

Structure-activity and binding characteristics

The structural and functional similarities of ORL1 receptor and classical opioid receptors do not necessary result in a pharmacological homology. Several lines of evidence indicate that none of the endogenous opioids, except DyA, bind to the ORL1 receptor. The existence of Phe residue at site 1 of NOFQ amino-terminal determines the binding to ORL1 receptor. Replacement of Phe1 with Tyr1 confers affinity at the opioid receptors leaving unchanged the binding affinity at ORL1 receptor (7). The concept of N-terminal "message" YGGF and C-terminal "address" LRRIRPKLK segments implied for DyA (13), despite outdated, proved useful for explanation of

Figure 3. *Amino acid sequence of NOFQ precursor**

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81                                                                  111
H L K R M P R V R S V V Q A R D A E P E A D A E P V A D E A D
H L R R M P R V R S L F Q E Q E E - P E P - - - - - - - - - -
112                                                                 142
E V E Q K Q L Q K R | F G G F T G A R K S A R K L A N Q | K R F G
E M E Q K Q L Q K R | F G G F T G A R K S A R K L A N Q | K R F G
143                                                                 173
G F T G A A R K S A R K L A N Q K R F S E F M R Q Y L V L S M
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** The numbers denote position in the amino acid sequence of precursor. Underlined in bold are shown the basic Lys-Arg motifs bounding the NOFQ molecule shown in rectangle. First line shows the sequence of rat precursor, second line shows the sequence of human precursor.*

high affinity and potency at ORL1 receptor of hexapeptide Ac-RYYRWK-NH2 obtained by a combinatorial scanning (74). Similarly, the removal of Phe1-residue in des-Phe-NOFQ resulted in a dramatic (over 2 000 fold) decrement (ED50 ≥ 1 μM) of affinity (7). It was found in studies of the affinity of NOFQ analogues obtained by alanine "scan" that Phe at sites 1 and 4 and Arg at site 8 were critical for binding (25). The authors reported that a 10-fold decrease in affinity resulted from the removal of Ser at position 10 and Lys at position 13. However affinity has not been significantly changed in Ala "scan" substitution. These findings indicate that N-terminal is more critical for binding than C-terminal with residues at sites 1, 4 and 8 being essential. The investigation of NOFQ C-terminal truncated fragments down to FGGFTG-NH2 have revealed a progressive decrement in affinity (IC50: NOFQ 13 ± 3 nM, FGGFTG 13400 ± 1730 nM) (25). The removal of Arg12 and/or Lys13 resulted in a total loss of activity (7). C-truncated NOFQ fragments down to FGGF displayed an ORL1 receptor agonist activity which was highest for fragments N1-16 to N1-13 and surprisingly N1-5 which IC50 was 520 ± 40 nM; lower was the activity of fragments N1-12 to N1-10 and N1-7; inactive were fragments N1-9 to N1-6. Similar studies have shown that N-truncated fragments N6-17 and N12-17 were potent ORL1 receptor agonists (25, 7, 93) while N2-17 was inactive (see above). The mouse vas deferens was reported a useful and simple *in vitro* assay for investigation of affinity and activity at ORL1 receptor (8). Utilising this tool in extensive studies of a series of NOFQ truncated fragments and analogues, Guerrini *et al* (34) concluded that basic residues of NOFQ amino acid sequence played an essential role for the interaction with ORL1 receptor. Taken together the data suggest that NOFQ activity towards ORL1 receptor is determined by the basic core of NOFQ molecule.

Metabolism

The elucidation of the pattern of NOFQ enzyme cleavage is indispensable for the complete understanding of its physiological role. This process could either terminate the action of the peptide or generate shorter fragments. Bearing in mind that behavioral effects of NOFQ are not opioid-like, Mogil *et al* (73) suggested that a wide spectrum of NOFQ effects could be due to generation of as yet still uncharacterised metabolites. The production of NOFQ fragments has been monitored by HPLC during incubation of mouse brain cortex punches in the absence or presence of selective endopeptidase inhibitors (76). The cleavage was time dependent and occured mainly at bounds Phe1-Gly2, Ala7-Arg8, Ala11-Arg12 and Arg12-Lys13. The larger were the amounts of the generated fragments N1-7, N1-11, N12-17, N13-17 and N2-17. Evidence has been also reported that zinc-containing proteolytic exofacial neutral aminopeptidase (51) cleaved (Km 1260 ± 300 μM) the bound Phe1-Gly2, whereas other bounds were most likely cleaved (Km 97 ± 8 μM) by the cytosolic endopeptidase 24.15 (1). This was confirmed *in vivo* by the use of a battery of selective inhibitors (84). In the human plasma, NOFQ biotransformation yielded mainly fragment N2-17 and minute quantities of fragments N1-12 and N1-8 (117). The quantification of NOFQ biotransformation by the enzyme activity isolated from cultured rat brain cortex cells, SH-SY5Y neuroblastoma and U1690 human lung carcinoma cell lines has been achieved by ESI MS coupled to SMART size-exclusion HPLC (108). Mass spectrometry (MS) analysis revealed as major products of NOFQ biotransformation fragments N1-9 and N1-13 which amounted as much as 5.49 ± 0.59 % RIC and 1.92 ± 0.31 % RIC, respectively. Minor component was the fragment N2-17. Less quantities were estimated for fragments N1-8, N1-12 and N1-15. The identification of fragments N10-13 and N10-17 suggested that N1-9 could be generated either from N1-13 or directly from NOFQ. The cleavage of peptide bounds positioned before, between or behind the basic Arg-Arg, Lys-Arg or Arg-Lys doublets was a characteristic feature of this NOFQ biotransformation pattern.

CELLULAR EFFECTS

The full repertoire of NOFQ induced cellular effects is still to be unravelled. The overall characteristics of all ORL1 receptor-mediated effects is Gi/Go transduction pathway which is a common transduction mechanism for all members of the superfamily of seven transmembrane domains G-protein coupled receptors.

Adenylyl cyclase

The net effect of NOFQ on adenylyl cyclase was investigated in LC132 transfected CHO cell line in two independent studies. The authors found that NOFQ was a potent inhibitor of forskolin-induced cAMP accumulation with an IC50 of 0.9 nM (69) and 1.05 nM (90). The maximal inhibition was 90% which was achieved at a concentration as high as 100 nM. The effect was blocked by BPT which implied that NOFQ inhibited adenylyl cyclase via a G-protein coupled mechanism. The same effect has been recently described in mouse brain homogenates (IC50 1 nM) (65) and NG 108-15 cell line (IC50 0.7 nM) (60).

Potassium channel and K^+ conductance

Strong evidence from patch clamp experiments indicated that NOFQ induced an outward current (45,66) with an EC50 of 12 nM. The current reversed polarity near the predicted K^+ equilibrium potential which suggested that NOFQ-induced outward current was due to an increased inwardly rectifying K^+ conductance (106) resulting in an impairment in cell excitability (67). The membrane currents in CA3 pyramidal cells which bear ORL1 receptor and GIRK channel were recorded by whole cell voltage clamp technique in mouse hippocampal slices (45). NOFQ induced outward current at -70 mV that was TTX insensitive. This is an indication that CA3 neurons but not other neurons in the slice have been the target of NOFQ action. The stimulation of ORL1 receptor activates GIRK channels that brings about K^+ outflux and consequent hyperpolarisation (45). It is worth mentioning that

GIRK channel is a transduction mechanism for both NOFQ and opioids despite these have opposite *in vivo* effects. Enkephalins could excite certain pyramidal neurons through hyperpolarisation of neighbour inhibitory interneurons which results from the opening of their GIRK channel without effect on neurons' membrane potential and resistance. On the other hand dynorphins could suppress CA3 pyramidal neurons by a presynaptic inhibition of the Mossy fibres. In a contrast to these effects NOFQ can directly change the electrical properties of the neuron thus inhibiting the cell firing (45).

Ca^{2+} homeostasis

NOFQ has been reported to modulate the activity of VGCC in various cell populations. The whole-cell patch clamp studies of freshly dissociated CA3 hippocampal neurons revealed that NOFQ inhibited Ca^{2+} channel currents in a concentration-dependent way with an EC50 of 98 nM. Three types of calcium channel (L, N and P/Q) were partially inhibited. The N-calcium cannel, that plays a central role in exocytotic release of fast transmitters, has been mostly affected (54). Ca^{2+} channel current was similarly inhibited in SH-SY5Y human neuroblastoma cells with an EC50 of 42 nM (18). Free intracellular Ca^{2+} concentration was not changed by NOFQ. However in the presence of carbachol NOFQ mobilised intracellular Ca^{2+} causing a swift and transient increase of the intracellular Ca^{2+} (18). The effects were blocked by BPT pretreatment that indicated G-protein dependent processes.

Neurotransmission

The cellular effects of NOFQ suggest an overall inhibitory action which has been most likely realised as a presynaptic inhibition of transmitter release. *In vivo* microdialysis by positioned in nucleus accumbens of anaesthetised rats probes revealed that NOFQ injected icv dose-dependently decreased DA release (77). The nadir inhibition was 47.8 % which was reached 60 min after injection without significant changes in the levels of glutamate, aspartate and GABA. The effect might be either due to a direct action on mesolimbic n. accumbens neurons or an indirect action via neurons located in the VTA. The opposite effect was reported by Konya *et al* (56). Employing similar methodological approach the authors found that NOFQ induced a naloxone-sensitive release of DA from striatum of conscious freely moving rats. Apparently more studies are needed to clarify *in vivo* as well as *in vitro* effects of NOFQ on DA neurotransmission. NOFQ was found to inhibit the release of glutamate induced by high K^+ (80) or glutamatergic ventral root potentials evoked by electrical stimulation of dorsal root in rat spinal cord preparation (28). NOFQ inhibited GABAergic transmission in the brain periaqueductal gray neurons (107) by a stronger pre- rather than postsynaptic action (59). Light-induced ACh release from the amacrine neurons in rabbit retina was similarly inhibited by NOFQ (78). Also, NOFQ-mediated inhibition of electric field stimulation-induced cholinergic and tachykinergic neurotrasmission was documented (88a).

Other cellular effects

A good deal of information is accumulating about the effect of NOFQ on the bioelectrical activity of spinal cord and brain neurons. The extracellular recording from dorsal horn neurons responding to Aβ-and C-fibre cutaneous afferents after transcutaneous electrical stimulation of the hind paw revealed that NOFQ applied intrathecally (ith) at doses of 5, 50 and 225 μg inhibited dose-dependently the C-fibre but not Aβ-fibre evoked wind-up and post-discharge (100). This finding contrasted with previously reported hyperalgesic action of NOFQ after a supraspinal application (69,90). The amplitude of EPSC recorded in a whole-cell patch-clamp configuration in dorsal root neurons was reversibly reduced by NOFQ with EC50 of 485 ± 47 nM that was an order of magnitude higher than EC50 for inhibition of Ca^{2+} channel current. Average maximum inhibition of 57.6 ± 5.7 % was achieved at concentrations higher than 3 μM. The peptide reduced mEPSC frequency with no effect on their amplitude (59). Data from previous investigation, similarly inconsistent with the hyperalgesic action of NOFQ, have shown that after microiontophoretic application into trigeminal nucleus caudatus which is a major way of nociceptive, thermoceptive and mechanoceptive afferentation, NOFQ produced 70.4 ± 3.69 % and 75.5 ± 9.72 % inhibition of NMDA- or AMPA-evoked response (total number of spikes per stimulus), respectively. The peptide produced a long-lived facilitation of NMDA-evoked responses (110). In brain periaqueductal grey matter (PAG), NOFQ produced a pair-pulse facilitation and inhibition of fast IPSC and EPSC in the half of neurons, while in ventromedial PAG it produced an inhibition of IPSC in the majority of neurons. NOFQ also decreased the frequency of mIPSC and mEPSC (107). Recently several lines of evidence have pointed to the strong action of NOFQ on the neuronal activity in hippocampus. The peptide inhibited concentration-dependently the population spikes in CA1 region and DG (30 ± 3 % and 16 ± 2 %, respectively). NOFQ reduced EPSP slope by 44 ± 7 %. This was associated with 11 ± 4 % increase in facilitation which might indicate that NOFQ depression of synaptic transmission was due to a presynaptic inhibition of the release of excitatory transmitter (118). It occurred most likely at synapses between Schaffer collaterals and CA1 pyramidal and DG granule neurons. The authors found that NOFQ (10 μM) abolished the induction of LTP in CA1. It was suggested that activation of ORL1 receptors in hippocampus can modulate the use-dependent synaptic plasticity involved in learning and memory. Such a notion was further supported by Manabe *et al* (63) who reported that ORL1 receptor-deficient mice showed a larger long-term potentiation in CA1 region than wild type control mice.

BEHAVIORAL EFFECTS

The first papers of isolation and amino acid sequencing endowed NOFQ with the algogenic activity. Following this initial observation it was revealed that NOFQ could functionally antagonise μ- and δ-opioid effects in different brain areas and pathways being endogenous pronociceptive mediator. However, later findings did not confirm but rather confront this notion enabling one to get confused by plethora of data about NOFQ-produced hyperalgesia, analgesia, absence of pain relief or reversal of opioid analgesia.

Nociception

The acute icv administration of NOFQ (10 ng to 80 μg per mouse) produced a dose related hyperalgesia evaluated in hot-plate (69) or tail-flick (90) test. The latter team reported no effect after ith administration of NOFQ. Utilising a flexor-reflex paradigm, Inoue *et al* (47) demonstrated an algogenic effect of NOFQ stronger more than 4 orders than the effect of SP. The authors suggested that NOFQ produced nociceptive responses through release of SP from peripheral nerve endings. Direct dose-dependent reduction of tail withdrawal latency after icv. administration of NOFQ was observed also in saline treated mice (9). In contrast, other authors have found monophasic hyperalgesia after ith administration of 5-50 ng/kg NOFQ which effect was antagonised by glycine (40). NOFQ produced a similar hyperalgesia and abolished gestational and steroid-induced antinociception after ith application in female nulliparous rats (22). Recently, NOFQ was found to produce a short-lasting nociceptive flexor response after local intraplantar injection in mice (47). The effect was attenuated by local injections of botulinum toxin, specific NK1 (substance P) but not NK2 (substance K) antagonists, and was abolished by injection of capsaicin. This was associated with NOFQ-induced local release of SP and consequent excitement of the sensory nerve endings. The authors reported that responses were completely lost in tachykinin 1 gene knockout ($tac1^{-/-}$) mice. The NOFQ increased pain perception can also evoke nociceptive responses to nonnoxious stimuli, i.e. allodynia (47a,86). The NOFQ allodynia was blocked by PGD_2 in a dose-dependent manner with an IC_{50} of 26 ng/kg (70). Studying the time-course of NOFQ action, Rossi *et al* (93) have found that icv injection of NOFQ produced hyperalgesia which was dose-dependent with a maximum at a dose of 10 μg. This effect gradually resolved over time and tail withdrawal latency has augmented progressively indicating transformation of the effect into analgesia. The peak analgesic effect was reached 30-45 min after administration. Several reports described an analgesic effect of NOFQ after ith administration in rats (104) which was naloxone-reversible (94) or naloxone-resistant (113). Antinociceptive affect verified by a dose-dependent reduction of flinching behavior in rat formalin test was observed after ith. administration of NOFQ via continuously implanted lumbar catheter (26). Antinociceptive effect of ith NOFQ on the inflammatory pain was demonstrated by attenuation of ipsilateral thermal hyperalgesia in carageenan-inuced rat hind paw edema (115). NOFQ ith injected alleviated dose-dependently the mechanical and cold allodynia-like behavior in models of neuropathic pain and heat hyperalgesia (39). The authors reported significantly lower antinociceptive efficacy of NOFQ in carageenan-induced thermal hyperalgesia. Antinociceptive effect of NOFQ was found in diabetic rats due to modulation of SP release at pre- or postsynaptic sites (49). The apparent discrepancy in the pro- and antipain action of NOFQ mirrors the complexity of its diverse physiological activity as well as the differences between used species, routes of administration and doses. It is plausible to suggest that at brain cortical or subcortical levels, NOFQ is a pronociceptive endogenous peptide, whereas at the spinal cord level it has antinociceptive activity. The latter is likely to involve the inhibition of SP- and CGRP-release from sensory nerve terminals (42), depression of Aβ-mediated component of glutamatergic population of ventral root potentials (59) and C-fibre wind-up phenomenon in dorsal horn (100). The best known analgesic drug is morphine which however brings about tolerance and dependence/addiction. The first involves intracellular mechanisms such as the receptor desensitisation and the latter changes the interneuron plasticity that depends on postsynaptic dopaminergic transmission (79). It has been reported that mice lacking NOFQ receptor gene showed a 50% loss of tolerance liability to morphine analgesia (105) which implied interactions of opioid and NOFQ processes at recognition or transduction levels. Such notion was reinforced by the findings that NOFQ antagonised morphine-induced analgesia in brain but potentiated it in the spinal cord (104). Most recent data showed that in tolerant rats upon continuous use of high doses of morphine the release and biosynthesis of NOFQ in PAG, amygdala and cerebroventricular perfusate were activated which might be a delayed negative feedback control on opioid analgesia (120).

It is well established that neurotrophins including nerve growth factor, brain-derived neurotrophic factor, and neurotrophin-3, -4/5 play a crucial role in the development and survival of nociceptive neurons (67a,96a). Furthermore, neurotrophic factors from other families such as glial cell-derived neurotrophic factor are also required to assure survival of certain classes of nociceptors (67a). Note that ciliary neurotrophic factor significantly increased NOFQ expression in brain cells (7a). Whether neurotrophic factors might be involved in nociception/antinociception mediated by NOFQ remains to be evaluated (see 31a).

Stress and anxiety

The conflicting results found in studies of NOFQ action on pain sensitivity have been associated with a large spectrum of diverse physiological reactions controlled eventually by the peptide. Indeed, it was found that NOFQ icv injected mice displaced equivalent pain sensitivity estimated in peritoneal and hot-plate tests to that of uninjected mice. However, vehicle-injected mice displayed a stress-induced analgesia (SIA), which was reversed by NOFQ (72). The authors concluded that apparent NOFQ-induced hyperalgesia was actually the reversal of SIA. Further studies have shown that NOFQ blocked completely systemic and supraspinal morphine antinociception produced via μ-, δ- or κ-opioid receptors (73). NOFQ was found to reverse also morphine-induced hypothermia and Straub-reflex. The NOFQ functional antagonism towards opioid action was realised via indirect mechanism which could involve most likely an as yet unidentified NOFQ metabolite (72). Similar studies have shown that ith NOFQ did not attenuated spinal analgesia after ith morphine application in mice (33). It was concluded that NOFQ acts as a supraspinal, but not as a spinal antiopioid peptide. The antagonism between NOFQ and opioid system could have considerable clinical utility as an affective adjunct in morphine pharmacotherapy, allowing lower doses of morphine (72). The abundance of NOFQ receptors in brain amygdaloid complex, central gray matter, raphe nuclei, as well as thalamus and hypothalamus, which participate in the integration of emotional components of fear and stress, suggested that NOFQ should control behavior responses related to states of anxiety. This has been reported recently by Jenck *et al* (48). The authors demonstrated the stimulation of spontaneous locomotion and exploration with low nonsedating doses (0.1-3 nmol) NOFQ which might be related to its anxiolytic action. Higher doses NOFQ have opposite effect resembling U-shaped fluctuations that are typical for the action of conventional anxiolytics (48). The authors concluded that NOFQ might play the role of a general modulator of acute behavioral responses to stress. The anxiolytic properties were associated with the presence of ORL1 receptors on the neurones in locus coeruleus, amygdala, central gray, thalamus and hypothalamus. An important finding was reported recently that in 98 % of the neurons in rat lateral amygdala, NOFQ brought about the impairment of cell excitability through activation of inwardly rectifying K^+ conductance (67) supporting the likely role of NOFQ in the reduction of fear responsiveness and stress. The response was sensitive to Ba^{2+}, prevented by G-protein inactivation and selectively blocked by NOFQ antagonist. It is known that other endogenous peptides, such as CCK, CRF or NPY, are similarly involved in the modulation of stress responses. CCK and CRF induce anxiogenic effects, whereas NPY is an anxiolitic (48). It seems that NOFQ is a major constituent of the apparently reciprocal peptidergic control of behavioral responses to stressful stimuli (48).

Memory and learning

Several lines of evidence showed that ORL1 receptors were densely distributed in the hippocampus. On the other hand it is a general consensus that memory and learning require the integrative control of the hippocampus over cognitive functions. Taken together these facts point out the likely role of NOFQ in memory processes and learning. Mutant mice lacking ORL1 receptor were found healthy, fertile, with no significant anatomical defects or alterations of nociception and thermoregulation (82) which implies for an insignificant role of NOFQ in the regulation of the related physiological systems. However, the mutant mice possess greater learning ability and have better memory (32). LTP in CA1 region was found significantly larger (158.4 ± 8.5 %) in the mutant ORL1 receptor-deficient mice (63) which suggested a significant role of ORL1 receptor in negative regulation of LTP induction. NOFQ could negatively modulate the synaptic plasticity in granule cells of dentate gyrus by a postsynaptic hyperpolarisation (119). It is plausible to suggest that specific NOFQ antagonists could be effective in the treatment of memory deficits. NOFQ was found to impair the spatial learning and decrease the exploratory behavior after a hippocampal microinjection in rats (95). The enhancement of spatial attention was demonstrated subsequently in ORL1 receptor knockout mice (62). The authors found also a concomitant decrease of DA content in the frontal cortex with a likely suggestion that NOFQ regulates stimulatively the dopaminergic system (62).

Locomotion and motivation

The study of NOFQ effect on locomotor activity has paralleled the investigation of its action on pain sensitivity. Injected icv in mice at a dose of 10 nmol/mouse (equal to 18 μg/mouse) NOFQ was found to decrease horizontal and vertical activity and lower the muscular tone in all mice. It induced ataxia and loss of righting reflex in 66 % of animals (90). A severe reduction in locomotor activity after icv administered NOFQ in mice was later measured in spontaneous locomotion and open field tests. The effect was potentiated by bestatin (84). Hypolocomotion, disruption of balance and motor control, atypical posture, wobbling from side to side and flaccid muscular tone with no changes in conditioned preference or aversion were observed after icv injection of NOFQ in rats at a dose of 100 nmol followed by a rapid development of tolerance (23). The opposite effects as the increase in horizontal and vertical locomotion and activation of exploratory behavior were observed in mice after icv administration of doses as lower as 10-100 ng/mouse, which has been attributed to NOFQ stimulation of dopaminergic transmission (30). On the other hand NOFQ induced suppression of dopaminergic neurotransmission in nucleus accumbens (77) implied that the peptide could produce aversive effects. However, NOFQ was found motivationally inert in rats (23). Being injected into lateral ventricle of opioid-dependent rats,

NOFQ failed to precipitate withdrawal symptoms (104). Further studies are needed to elucidate the role of NOFQ in motivation and withdrawal syndrome.

Feeding and neuroendocrine functions

The stimulation of feeding behavior by agonists of opioid receptors, the most potent being κ-agonists, is firmly established (19). The structural similarity of NOFQ to DyA and ORL1 receptor to κ-opioid receptor from the one hand and highest level of NOFQ and ORL1 receptors in the appetite controlling areas of hypothalamus from the other, implied strongly that NOFQ could operate as an endogenous orexigenic peptide. Indeed, it was found that injected via intraventricular cannula in satiated rats NOFQ induced a considerable increase of food intake in the first hour following injection (89). NOFQ induced similar stimulation of food intake by administration into nucleus accumbens or hypothalamic ventromedial area (101). Unlike other this NOFQ effect was blocked by naloxone similarly to inhibition of orexigenic acton of the nonopioid peptide NPY. It is unlikely that naloxone antagonism of NOFQ stimulated food intake is stress-related (89) since analgesia and hyperphagia which are both stress-induced phenomena should be reversibly controlled. Recent studies of Leventhal *et al* (58) add a further support to NOFQ control of orexigenic behavior. Rats with implanted cannulas were given icv ORL1 receptor antisense or missense probes. It was found that NOFQ-induced hyperphagia was significantly diminished and eating latency reduced in antisense probe treated rats. The effects were specific since no changes in food intake were observed in rats receiving missense probe. For the time being few are the data available about the action of NOFQ on endocrine system. It was found that icv administration of NOFQ at doses as high as 12.5 μg/rat enhanced circulating growth hormone with no influence on prolactin and corticotropin levels (5). Inhibition of excitability of electrically identified oxytocin and vasopressin supraoptic hypothalamic neurons was revealed by voltage clamp technique (24). NOFQ was found to inhibit spontaneous discharge in 45 % of neurons tested and diminish firing rate with a induction of repetitive burst firing. The inhibition was identical for oxytocin and vasopressin neurons. The authors concluded that NOFQ action was mediated via postsynaptic ORL1 receptors.

Other behavioral effects

The brain stem periolivary nuclei serve as a central auditory subcortical relay station which is enriched of ORL1 receptor mRNA. Mice lacking ORL1 receptor cannot adapt to intense acoustic challenges (82). The nature of this action remains hitherto to unravel. It is known that ORL1 receptor and NOFQ mRNA were extensively distributed in medial vestibular nucleus of rat brain stem. Investigations *in vitro* demonstrated that NOFQ dose-dependently inhibited 86 % of spontaneously active neurons which was antagonised by a specific ORL1 receptor antagonist. *In vivo* NOFQ induced a prolongation of postrotatory nystagmus in darkness (102). Recently, data have been presented that NOFQ administered at lower doses increased temperature sensitivity of warm-sensitive neurons of the preoptic area of anterior hypothalamus. At higher doses NOFQ decreased the temperature sensitivity (114). In *in vivo* experiments, the authors demonstrated that following intrahypothalamic application NOFQ induced an increase of temperature sensitivity which coincided with a decrease of body temperature. The effects of NOFQ were inhibited by specific ORL1 receptor antagonists, but not by specific opioid receptor antagonists.

PERIPHERAL EFFECTS

Immune competent cells

There are still few investigations on the role which ORL1 receptor and NOFQ could play in immune reactions. It was shown earlier that expression of ORL1 receptor in human lymphocytes augmented several folds upon activation with phytoagglutinin (112). The authors reported that ORL1 receptor specific antisense nucleotide suppressed strongly the production of M- and G- immunoglobulin which coincided with concomitant inhibition of lymphocyte proliferation (38). These findings together with the presence of ORL1 receptor on the membrane of peripheral and splenic lymphocytes indicate the participation of NOFQ in immune regulation albeit through an yet unknown mechanism. This viewpoint was not supported by other investigators who did not observe abnormalities in bone marrow and blood lymphocytes of ORL1 receptor-deficient rats (82). Recent report suggested that NOFQ may stimulate histamine release from mast cells (53a).

Urinary system

Following iv (10 nmol/kg/min) or icv (1 to 30 μg/rat) administration of NOFQ the urine flow markedly increased and the urinary sodium excretion decreased (50). This finding implied strongly that NOFQ could penetrate the blood-brain barrier. Most recently *in vivo* investigations revealed that NOFQ produced a dose-dependent transient suppression of distension-induced micturition reflex (36). The peptide inhibited the electrically evoked neurogenic bladder contractions. NOFQ was found to abolish the reflex response to locally applied capsaicin without effect on ATP- or acetylcholine-induced bladder contractions. It was suggested that NOFQ inhibited the micturation most likely by a suppression of transmitter release from postganglionic nerve terminals or by an inhibition of bladder afferent nerves (36).

Smooth muscle

Several visceral smooth muscles were found sensitive to NOFQ action depending on organ or species characteristics. The peptide did not stimulate the motor activity of rabbit and mouse vas deferens or guinea pig ileum. However, NOFQ produced a concentration-dependent inhibition of electrically induced contraction of mouse vas deferens by 76 ± 4 % with pIC_{50} 7.88 and guinea-pig ileum by 48 ± 6 % with pIC_{50} 8.12 (8). The authors found NOFQ ineffective on the contraction of rabbit vas deferens and the relaxation of guinea pig ileum. NOFQ inhibited the electrically induced nonadrenergic, noncholinergic contractions of guinea pig isolated main bronchi and did not affect capsaicin induced contractions (96). It caused 59 ± 11 % decrease of electrically induced release of SP, which may indicate a NOFQ role in the regulation of release of sensory peptides. Indeed, data have been presented that NOFQ at a concentration of 100 nM caused 50 % inhibition of guinea-pig bronchial contractions evoked by 5 Hz electrical stimulation (29; also see 88a). The effect was most likely due to NOFQ prejunctional release from the NOFQ immunoreactive nerve fibres identified as its endogenous neurogenic source. However, NOFQ produced in rat colon concentration-dependently early tonic contraction which was followed by rhythmic slow waves. Injected iv in anesthetised rats, NOFQ evoked phasic contractions of proximal colon (103) much likely to the effect of morphine. However, the rate of intestinal transit was accelerated dose-dependently by NOFQ and significantly retarded by morphine.

Cardiovascular system

In general, NOFQ decreased the tone of vascular smooth muscle cells and dilated the blood vessels. Isolated rings of feline renal, mesenteric, carotid and femoral arteries with intact endothelium relaxed concentration-dependently by NOFQ (37). The same vasodilator effect of NOFQ was revealed in isolated pressurised resistance arteries from rat mesenteric vascular bed. NOFQ increased arterial diameter concentration-dependently by a direct vasodilator mechanism (12). *In vivo* bolus iv administration of NOFQ in unanesthetised normotensive mice caused a sharp decrease of mean arterial blood pressure and heart rate (61). The authors found that NOFQ at a dose of 100 nM/kg decreased mean arterial blood pressure from 114 ± 3 to 97 ± 2 mm Hg and reduced heart rate from 542 ± 43 to 479 ± 31 beats/min by a concomitant increase of aortic blood flow by 41 ± 5 %. The NOFQ evoked decrease of arterial blood pressure and fall of heart rate coincided with a profound inhibition of spontaneous discharges of neurons in rostral ventrolateral medulla in isolated rat brain slices (16), which has been completely antagonised by specific NOFQ antagonists (17). Injected locally in the same nucleus of anesthetised rats NOFQ evoked a similar decrease of arterial blood pressure and heart rate. Taken together the data suggested that NOFQ could induce powerful inhibition of the medullar integrative centre of cardiovascular control. In an extensive study, Champion *et al* (10) reported that NOFQ dilated the hindquarter vascular bed and decrease the perfusion pressure in rats. The effect was not changed by selective inhibition of NOS which indicated that NOFQ action was not mediated by the nitroxidergic mechanism. Further, intradermal administration of NOFQ increased vascular permeability via histamine release from mast cells (53a).

Other behavioral effects

NOFQ produced a swift sustained penile erection in anesthetised cats (4). The authors compared the erectile effect of NOFQ and other erection-producing substances as CGRP, SP, SNP and PGE1 with the effect of a standard triple-drug cocktail of 1.65 mg papaverine, 25 μg phentolamine and 0.5 μg PGE1. It was found that intracavernosal application of NOFQ produced penile erection comparable to erection produced by the triple-drug combination. The erectile effect was found dose-dependent with intracavernosal applications of NOFQ or its Tyr1-analogue in anesthetised cats in a dose range of 0.3 - 30 nM (11).

ANTAGONISTS AND ANALOGUES

The isolation of NOFQ from brain tissue raised the problem of whether a natural NOFQ antagonist did exist. This triggered intensive studies which in a short time culminated in the disclosure of four processing products delineated between four potential cleavage sites in the amino acid sequence of prepronociceptin (87). The preproNOFQ fragments 117-132 (bovine and human), 122-135 (mouse) and 116-132 (rat) represent a heptadecapeptide (Fig. 4), which specifically blocks NOFQ effects, hence termed nocistatin (NST) (87). NST blocked NOFQ-induced allodynia with ID_{50} of 715 fg (range 34 fg - 4.25 pg). It attenuated significantly NOFQ-induced hyperalgesia. Interestingly, NST also inhibits PGE_2-induced allodynia and hyperalgesia. The authors subjected to immunoaffinity chromatography and reverse-phase HPLC the crude extract of bovine brain to trace eventually the endogenous NST. A single peak of absorbance was disclosed at 214 nm which eluted after 36.9 min. It coincided with the immunoreactivity detected by anti-bPNP-3 antibody. The amino acid sequence was verified as Thr^{1}-Glu-Pro-Gly-Leu-Glu-Glu-Val-Gly-Glu-Ile-Glu-Gln-Lys-Gln-Leu-Gln^{17} being identical to the amino acid sequence of bPNP-3. The peptide bounded with a high affinity to the membrane fractions obtained from brain and spinal cord preparations.

Figure 4. *Amino acid sequence of prepronociceptin fragment bearing nociceptin and nocistatin sequences*

Amino acid residues and position

	bPNP-3 (NOCISTATIN)	NOCICEPTIN	bPNP-4
BOVINE(110) R	TEPGLEKVGEIEQKQLQ	KR FGGFTGARKSARKLANQ	KF FSEFMRQYLVLSMQSSQ RRF
HUMAN (110) E	PEPGMEEAGEMEQKQLQ	KR FGGFTGARKSARKLANQ	KF FSEFMRQYLVLSMQSSQ RRF
MOUSE (121) D	AEPGADDAKEVEQKQLQ	KR FGGFTGARKSARKLANQ	KF FSEFMRQYLVLSMQSSQ RRF
RAT (115) D	AEPVADEADEVEQKQLQ	KR FGGFTGARKSARKLANQ	KF FSEFMRQYLVLSMQSSQ RRF

The *in vitro* studies have revealed that NOFQ-induced decrease of glutamate release was fully reversed by NST at a concentration of 100 nM (81). Recently, human NST was estimated about 10 times less potent that bovine NST to block NOFQ- or PGE2-induced allodynia in mice with a ID_{50} of 329 pg/kg and 16.6 ng/kg, respectively (71). Further investigations are needed to elucidate the localisation, distribution, receptor affinity/specificity and potency of NST in various species. The lack of other than NST selective NOFQ antagonists impedes the progress in the pharmacological analysis of ORL1 receptor. Carbetapentane and rimcazole were two compounds endowed with antagonistic properties which have been described as ORL1 receptor antagonists (55). However they showed less affinity for ORL1 receptor. The compound recently found a selective and potent *in vitro* ORL1 receptor antagonist was [$Phe^1Y(CH_2$-NH)Gly^2]NC(1-13)NH_2 (35). It antagonised the inhibitory effect of NOFQ on electrically induced contractions of mouse vas deferens and guinea-pig ileum isolated preparations with a pA2 value of 6.75 and 7.02, respectively. Most recent data confirmed that NC (1-13) at concentrations above 3 mM antagonised the inhibitory effect of NOFQ on the spike discharges of individual units in rostral ventrolateral medulla region (17). No interaction with other opioid receptors was observed. In *in vivo* studies NC (1-13) prevented in unanesthetised mice NOFQ-induced hypotension and bradycardia and increased aortic flow without an effect on basal mean arterial blood pressure or heart rate (61). In a sharp contrast to these results, Butour *et al* (7) reported that in preparations of crude membrane fraction of CHO cells expressing ORL1 receptor, NC (1-13) is a potent inhibitor of forskolin-induced accumulation of cAMP being almost equipotent to NOFQ. The analogues NOFQ(NH_2) and N1-13(NH_2) mimicked the effect of natural NOFQ and N1-9(NH_2) were inactive in reducing tail withdrawal latency or in preventing morphine analgesia (9).

CONCLUSION

The study of NOFQ is still in its infancy. A plausible hypothesis suggests that NOFQ is an important modulator of vital physiological processes which is likely to operate presynaptically. In keeping with this notion is the presence of ORL1 receptors on interneurons in many CNS areas and peripheral organs: (*i*) cortical and limbic: cortex, amygdala, hippocampus, habenula, septum, (*ii*) hypothalamus: paraventricular and ventromedial nuclei, (*iii*) brain stem: locus coeruleus, central grey matter, dorsal raphe and parabracheal nuclei, (*iv*) spinal cord: ventral and dorsal horn, dorsal root ganglion, (*v*) visceral organs: intestine, liver, spleen, vas deferens, lung, (*vi*) skeletal muscles, and (*vii*) lymphocytes and mast cells. This implies that NOFQ might be involved in (*i*) learning and memory, (*ii*) attention and emotions, (*iii*) muscular tone and movements, (*iv*) endocrine functions, (*v*) visual, auditory, olfactory, gustatory, and nociceptive sensory perceptions, (*vi*) airway and cardiovascular physiology, and (*vii*) immune reactions. For the time being much attention is paid to NOFQ modulation of nociception (21,43). It is an axiomatic knowledge that morphine is the drug of first choice in severe pain. However, deleterious complications have been frequently precipitated with its usage. The activation of NOFQ system does not induce most of morphine unwanted effects. Therefore NOFQ antagonist NST might be the first of a new class of centrally-acting medicines suitable for treatment of injury-associated pain (64). In conclusion, NOFQ resembles chemically opioid peptides being functionally not a proper opioid peptide, while ORL1 receptor is homologous to opioid receptors being distinct from all classes of known opioid receptors. ORL1 receptor and its endogenous agonist NOFQ may therefore represent a quite distinct peptide regulatory system in the vertebrates.

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