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Review

Pain transmission regulated by novel neuropeptides nocistatin and nociceptin/orphanin FQ

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A neuropeptide nociceptin or orphanin FQ (Noc/OFQ) was previously identified as an endogenous ligand for the orphan opioid receptor-like receptor. Studies on the analysis of the precursor of Noc/OFQ, we identified another novel neuropeptide and named it "nocistatin". Noc/OFQ is involved in a broad range of pharmacological actions in various tissues from the central nervous system to the periphery. We found that intrathecal administration of Noc/OFQ induced pain responses including touch-evoked pain (allodynia) and thermal hyperalgesia. While both the allodynia and the hyperalgesia evoked by Noc/OFQ were transmitted by capsaicin-sensitive primary afferent fibers and initially in the disinhibition of the inhibitory glycinergic response, the allodynia was mediated by glutamate through the N-methyl-D-asparateate receptor comprising the GluRε1 subunit and the thermal hyperalgesia was mediated by substance P. Two bioactive peptides, Noc/OFQ and nocistatin exist the same precursor prepronociceptin/orphanin FQ. Simultaneous administration of nocistatin blocked the allodynia and thermal hyperalgesia induced by Noc/OFQ, and anti-nocistatin antibody decreased the threshold for the Noc/OFQ-induced allodynia. The carboxy-terminal hexapeptide of nocistatin (Glu-Gln-Lys-Gln-Leu-Gln), which is conserved in bovine, human and murine species, possesses the blocking activity. Nocistatin also attenuated the allodynia and thermal hyperalgesia evoked by prostaglandin E_2 and the inflammatory pain induced by formalin or carrageenan/kaolin, and reversed morphine tolerance and the Noc/OFQ-induced inhibition of morphine analgesia. Nocistatin and Noc/OFQ are novel bioactive peptides produced from the same precursor, and they play opposite roles in the regulation of pain transmission.

1, Introduction

Under physiological conditions, nociceptive signals evoked by noxious thermal, mechanical, and chemical stimuli are transmitted to the central nervous system (CNS) via activated nociceptors in the periphery. In contrast to acute pain, pathophysiological conditions of chronic pain states, which are often associated with prolonged tissue damage or injury, give rise to hyperalgesia and allodynia (2, 29). Hyperalgesia is defined as

an increased response to stimuli that are physiologically painful, whereas allodynia is defined as pain due to innocuous stimuli that do not normally provoke pain. The pathophysiology of pain involves a very complex interaction of many different peripheral and central structures. The dorsal horn of the spinal cord is an important site for pain transmission, and it constitutes the first relay station for incoming somatosensory information. Primary afferent nerve fibers including nociceptive Aδ, Aβ and C-fibers terminate in spinal dorsal horn. Modification of synaptic strength between theses afferents and second order neurons in spinal cord is considered a cellular mechanism of some forms of hyperalgesia and allodynia. In the spinal dorsal horn, the excitatory neurotransmitters, e.g. glutamate and substance P, released from the terminals induce the excitability of spinal neurons. On the other hand, serotonergic and noradrenergic descending pathways from the brainstem exert a strong inhibitory effect on dorsal horn transmission. Morphine and endogenous opioid peptides such as endorphins, enkephalins, and dynorphins produce their analgesic effects by modulating pain pathways through specific membrane receptors. Using molecular screening methods based on opioid receptor gene sequence, an orphan G protein-coupled receptor was identified (12). Furthermore, studies on the "orphan" receptor led to the discovery of a novel neuropeptide nociceptin/orphanin FQ (Noc/OFQ) (11, 20, 24) and nocistatin (21). This review describes recent advances in studies on Noc/OFQ and nocistatin, and particular attention will be paid to pain transmission at the level of the spinal dorsal horn.

2, Noc/OFQ and its receptor

Pharmacological studies have established that there are at least three major types, μ, δ and κ of opioid receptors. Molecular cloning of the δ-opioid receptor was succeeded in 1992, and followed by the cloning of the μ and κ-opioid receptors. These receptors inhibit adenylate cyclase, inactivate voltage-gated Ca^{2+} channels, and stimulate an inwardly rectifying K^+ conductance through a pertussis toxin-sensitive G-protein (10, 25). cDNA cloning studies have revealed a novel G-protein-coupled receptor with a high degree of amino acid sequence homology (approximately 60%) to the μ-, δ-, and κ-opioid receptors. This opioid receptor homologue, designated as ORL_1 in humans, as ROR-Ç and LC132 in rats, and as KOR-3 in mice, did not bind any of the known natural and synthetic opioid ligands and remained an "orphan" (12). A heptadecapeptide Noc/OFQ was isolated from rat, porcine, and bovine brains as an endogenous ligand for this "orphan" receptor on the basis of its inhibition of forskolin-induced cAMP accumulation in cells expressing these receptors (11, 20, 24). Noc/OFQ is structurally similar to dynorphin A, but structurally lacks the N-terminal tyrosine essential for opioid peptides to activate the receptor.

3, Pain transmission of Noc/OFQ

Noc/OFQ and its receptor are densely expressed in the superficial dorsal horn of the spinal cord. We found that the intrathecal (*i.t.*) administration of Noc/OFQ was shown to induce hyperalgesia to noxious stimuli by the hot plate test (3) and allodynia to innocuous tactile stimuli (3, 20). Noc/OFQ-induced allodynia was blocked by glycine, an N-methly-D-asparateate (NMDA) receptor antagonist, a non-NMDA

receptor antagonist, and a soluble guanylate cyclase inhibitor. Noc/OFQ-induced hyperalgesia was inhibited by glycine only. Noc/OFQ-induced allodynia and hyperalgesia may involve a common neurochemical event beginning with the disinhibition of the inhibitory glycinergic responses (3).

Activation of primary afferent C-fibers give rise to spinal release of substance P and glutamate, and these mediators facilitate the cascade of nociceptive processing. We designated experiments to elucidate the pathway and mediators of Noc/OFQ-evoked pain responses (14). Neonatal capsaicin treatment eliminated the induction of hyperalgesia and allodynia by Noc/OFQ. Whereas this treatment markedly reduced the content of substance P in the spinal cord, it did not affect the Noc/OFQ content or the expression of Noc/OFQ receptors and GluRε and GluRζ subunits of NMDA receptor. The substance P antagonists CP96,345 and CP99,994 blocked the Noc/OFQ-induced hyperalgesia, but not allodynia. In contrast, the Noc/OFQ-evoked allodynia, but not hyperalgesia, disapperared in NMDA receptor GluRε1 subunit knockout mice. Taken together, these results demonstrated that capsaicin-sensitive primary affenrent fibers are involved not only in the thermal hyperalgesia but also in tactile allodynia induced by Noc/OFQ, but in different pathways; the former is mediated by substance P and the latter is mediated by glutamate through the NMDA receptor comprising the GluRε1 subunit. Inoue et al. showed that intraplanter injection of Noc/OFQ evoked behavioral responses in a flexor-reflex paradigm (7). This response was suppressed by neonatal capsaicin pretreatment, NK_1 receptor antagonist, or in tachykinin gene knockout mice. Consistent with our results of hyperalgesia, Noc/OFQ involved in primary afferent C fibers via substance P release.

Noc/OFQ is reported to have different effects including nociception, no effect, and analgesia, depending on animal speicies tested, doses, routes of administration, and so on. Noc/OFQ is also known to have many central effects including locomotion, neuroendocrine secretion, and memory and/or learning. Recent reviews provide details on central roles of Noc/OFQ (8, 12).

4, Discovery of a novel bioactive peptide nocistatin

Noc/OFQ is processed from its precursor prepronociceptin/orphanin FQ (ppNoc/OFQ) (Fig. 1a), and its sequence is bounded by pairs of basic amino acids Lys-Arg, a general cleavage site for precursor maturation. We and several laboratories have determined the primary structures of bovine, human, mouse, and rat ppNoc/OFQ (6, 11, 19, 21, 23), and the amino acids sequence of ppNoc/OFQ are highly conserved among species. Further, the ppNoc/OFQ gene displays organizational and structural features that are very similar to those encoding precursors of the endogenous opioid peptides preproenkephalin, preprodynorphin, and preproopiomelanocortin, (15). These opioid peptide precursors possess several bioactive peptides (Fig. 1a). The ppNoc/OFQ comprises other putative cleavage sites. This prompted us to investigate the possibility that the Noc/OFQ precursor may comprise not only Noc/OFQ but also an additional bioactive peptide(s) as a maturation product(s).

The bovine ppNoc/OFQ protein consists of 176 amino acid residues. It contains three additional potential cleavage sites, which

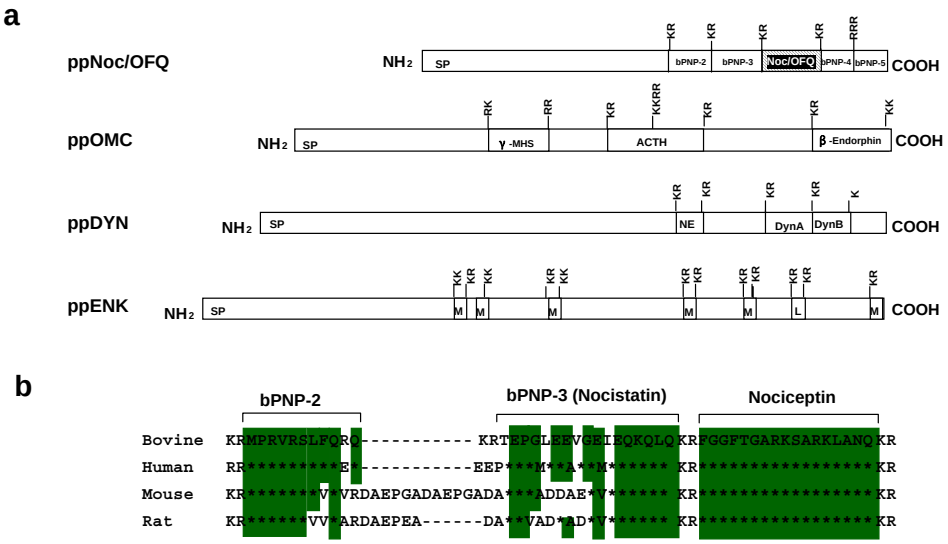

Figure 1 **(a)** Schematic representation of the structure of opioidpeptide precoursors. ppNoc/OFQ, preproNoc/OFQ; ppMOC, preproopiomelanocortin; ppDYN, preprodynorphin; ppENK, preproenkephalin; SP, signal peptide; γ-MSH, γ- melanocyte stimulating hormon; ACTH, adrenocorticotropic hormone; NE, neoendorphin; DynA, dynorphin A; DynB, dynorphin B; M, Met-enkepharin; L, Leu-enkepharin. bPNP-2, bPNP-3, bPNP-4 and bPNP-5 are putative peptides produced from bovine ppNoc/OFQ. **(b)** Alignment of deduced amino acid sequences among bovine, human, mouse, and rat nocistatin and Noc/OFQ. The conserved amino acids are shaded, and the putative proteolytic cleavage motifs are boxed.

would delineate four processing products (bPNP-2, bPNP-3, bPNP-4 and bPNP-5), as shown in Fig. 1a. To clarify whether these putative peptides produced from ppNoc/OFQ are biologically active *in vivo*, we synthesized them chemically and investigated *i.t.* injection of them effcts on pain responses (Fig. 2a). Although bPNP-3 (500 pg/mouse) did not induce allodynia by itself, the allodynia caused by *i.t.* injection of Noc/OFQ (50 pg/mouse) was significantly blocked by bPNP-3. The *i.t.* administration of bPNP-3 (500 pg/mouse) also attenuated the hyperalgesia evoked by Noc/OFQ (50 pg/mouse) in the hot plate test (Fig, 2b). So, we termed the bPNP-3 "nocistatin", after its apparent analgesic effects on nociception induced by Noc/OFQ.

Furthermore, in order to examine whether the endogenous nocistatin participates in pain transmission in the spinal cord, we examined the effect of pretreatment with anti-nocistatin antibody (antibody against mPNP-3) on the Noc/OFQ-induced allodynia (Fig. 2c). The dose-dependency of Noc/OFQ-induced allodynia in mice without pretreatment showed a bell-shaped pattern in the dose-range from 1 to 100 pg. The dose-response curve was shifted to the left by 2.5 orders at the half-maximal effective dose by *i.t.* pretreatment of mice with the antibody to nocistatin, but not with normal IgG. This result strongly suggests that the endogenous mPNP-3-immunoreactive peptide, possibly mouse nocistatin, plays an inhibitory role in Noc/OFQ-evoked allodynia in the spinal cord. In fact, both Noc/OFQ and nocistatin immunoreactivities were most abundant in the superficial laminae of mouse spinal dorsal horn (21).

5, Structure-activity relationship of nocistatin

Although bovine nocistatin (Thr[1]-Glu-Pro-Gly-Leu-Glu-Glu-Val-Gly-Glu-Ile-Glu-Gln-Lys-

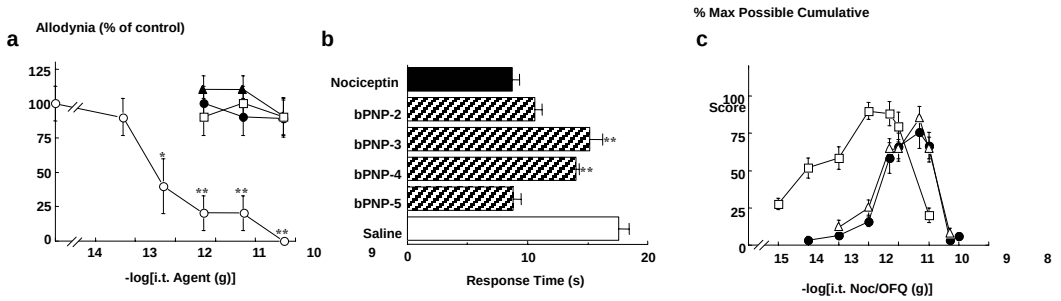

Figure 2 Effects of putative peptides deduced from bovine ppNoc/OFQ on allodynia (**a**) and hyperalgesia (**b**). Noc/OFQ (**a,** 50 pg/mouse) was simultaneously injected *i.t.* with various doses of bPNP-2 (●), bPNP-3 (○), bPNP-4 (Δ) or bPNP-5 (□). **b**, Noc/OFQ (50 pg) was injected without or with 500 pg of the indicated peptide. Each point represents the mean ± s.e.m. (n=6-10). *p<0.05, **p<0.01, versus nociceptin-injected group. **c,** Effect of *i.t.* pretreatment with anti-nocistatin antibody on Noc/OFQ –induced allodynia. Mice were not pretreated (●) or pretreated *i.t.* with 10 μg of anti-mPNP-3 IgG (□) or 10 μg of normal rabbit IgG (Δ) 1 h before *i.t.* injection of various doses of nociceptin. The allodynic score (mean ± s.e.m., n=6) is expressed as a percent of the maximum possible cumulative score over a 50-min period. Reprinted from Peptide, 21, Okuda-Ashitaka E. and Ito S., Nocistatin:a novel neuropeptide encoded by the gene for the nociceptin/orphanin FQ precursor., 1101-1109, 2000, with permission from Elsevier Science.

Gln-Leu-Gln[17]) is flanked by pairs of Lys-Arg at positions 109-110 and 128-129, the human, rat, and mouse precursors are devoid of the former cleavage site, resulting in processed products of 30, 35, and 41 amino acid residues, respectively (Fig. 1b). While this appears to delineate amino acid sequences of variable length and of low conservation among species, the variation of length among peptides is due to the repetition of a six-amino acid motif, Asp-Ala-Glu-Pro-Gly-Ala, and its divergent form: three times in mouse, twice in rat, and once in human, and the carboxyl-terminal half of these peptides is fairly conserved among species. We identified mature forms of NST in bovine, human, rat, and mouse brains (9). All of rat, mouse, and human putative peptides suppressed the Noc/OFQ-induced allodynia. Although pentapeptides Glu-Gln-Lys-Gln-Leu (PNP-3-6PΔC) and Gln-Lys-Gln-Leu-Gln (PNP-3-6PΔN) did not affect the Noc/OFQ-induced allodynia at 500 pg/mouse, a hexapeptide Glu-Gln-Lys-Gln-Leu-Gln (PNP-3-6P), which is 100 % conserved among species inhibited the Noc/OFQ-induced allodynia. These results demonstrate that these processed parts of the Noc/OFQ precursors showed similar biological activities beyond the species and that the carboxyl-terminal consensus portion is important for exhibiting the activity. Although the structural and conformational constraint for Noc/OFQ activation is most stringent in the amino-terminal part (positions 1-5, Phe-Gly-Gly-Phe-Thr) of the Noc/OFQ molecule, the carboxyl-terminal part (positions 12-17, Glu-Gln-Lys-Gln-Leu-Gln) appears to be the minimal essential core present within the nocistatin sequence.

6, Pain transmission of nocistatin

Allodynia

The *i.t.* administration of nocistatin attenuated the allodynia caused by *i.t.* injection of Noc/OFQ. Furthermore, *i.t.* administration of prostaglandin (PG) E_2 induces allodynia in mice (13, 27). Nocistatin also blocked the PGE_2-evoked allodynia in mice (21).

Thermal hyperalgesia

The *i.t.* administration of nocistatin attenuated the thermaol hyperalgesia caused by *i.t.* injection of Noc/OFQ and PGE_2 (21).

Inflammatory hyperalgesia

The formalin test is widely used as a model of clinical pain states. Subcutaneous (*s.c.*) injection of formalin produces biphasic pain behaviors: a first transient phase (0-5 min) ascribed to the direct effect of formalin on nociceptors and a second prolonged phase (10-30 min) related to the development of inflammation and central sensitization. The *i.t.* administration of nocistatin significantly inhibited the first phase and second phase of 2% formalin-evoked pain behaviors at 1 pg and at 10-1000 pg, respectively, in mice (17). Consistent with the results in mice, *i.t.* administration of nocistatin (3-30 μg/rat) attenuated the 5% formalin-induced flinching behavior in the first phase (29). On the other hand, *i.t.* Noc/OFQ at high doses of 0.3-3 μg/mouse significantly inhibited both phases of 2% formalin-induced pain, but it aggravated the 1% formalin-induced second phase at low dose of 10 pg/mouse. The latter aggravating effect of Noc/OFQ was completely blocked by *i.t.* administration of 10 pg nocistatin, but the former inhibitory effect of 1 μg/mouse Noc/OFQ was not reversed by 1 μg nocistatin in mice. In this connection, administration of nocistatin (30 g/rat) failed to block the analgesic effect of Noc/OFQ (30 μg/rat) in the rat formalin test (30), suggesting that the action mechanisms of Noc/OFQ at low and high doses are different (8, 22).

Although the *i.c.v.* administration of nocistatin induced neither hyperalgesia nor analgesia by itself, nocistatin (0.5-50 pmol/rat) dose-dependently reduced the inflammatory hyperalgesia induced by hindlimb intraplantar injection of carrageenan/kaolin in the rat paw-pressure test (16).

Morphine analgesia and tolerance

The *i.c.v.* injection of nocistatin (0.05-500 ng/rat) also reversed the anti-morphine analgesia of Noc/OFQ (8 μg/rat) in the rat tail-flick test, but nocistatin did not affect basal tail-flick latency or morphine analgesia (32). Furthermore, the *i.c.v.* administration of Noc/OFQ antibody reversed morphine tolerance, and the release of Noc/OFQ was increased during morphine tolerance. Both chronic and acute morphine tolerance were reversed by the *i.c.v.* injection of nocistatin (26).

Glutamate release

The inhibition of K^+-evoked glutamate release by Noc/OFQ was fully reversed by nocistatin in rat brain slices, while nocistatin had no effect on the K^+-evoked glutamate release alone (18). In vivo, the *i.c.v.* administration of Noc/OFQ shortened the step-down latency and impaired spontaneous alternation in the Y-maze test in mice. Although there was no significant effect of nociatatin by itself, nocistatin counteracted the impairment of learning and memory induced by Noc/OFQ (4). Furthermore, the impairment of learning and memory induced by scopolamine, a muscarinic cholinergic receptor antagonist, was reversed by the *i.c.v.* administration of nocistatin (5). Nocistatin is involved in not only pain transmission but also learning and memory.

7, Cellular mechanisms of nocistatin and Noc/OFQ

Although nocistatin inhibited the pharmacological functions induced by Noc/OFQ, nocistatin neither displaced [^{3}H]Noc/OFQ binding nor attenuated the inhibition of forskolin-induced cAMP accumulation by Noc/OFQ in cells transfected with the receptor. At the level of the spinal cord dorsal horn neuron,

nocistatin selectivity reduces inhibitory glycinergic and GABAergic synaptic transmission but leaves excitatory glutamatergic transmission unaffected, whereas Noc/OFQ only interferes with glutamatergic transmission (31). Although this inhibition by Noc/OFQ on excitatory transmission is completely absent in mice lacking Noc/OFQ receptor, this action of nocistatin on inhibitory transmission is completely retained in the mutant mice (1). These findings suggest the presence of a NST-specific receptor. The cDNA cloning of nocistatin receptor and investigation of its signal transduction are in progress in our laboratory.

8, Conclusions

Although preproenkephalin, preprodynorphin, and preproopiomelanocortin contain multiple repetitive peptides with related or distinct functions, nocistatin and Noc/OFQ generated from the same precursor ppNoc/OFQ, play opposite roles in the CNS. Both nocistatin and Noc/OFQ exist widely in the human, bovine, and rodent nervous systems. The function of nocistatin and Noc/OFQ may be regulated at multiple steps; synthesis and release of these peptides, their degradation and/or removal, and receptors. The reserch of nocistatin receptor and its release may lead to the design of a novel analgesic devoid the addiction and tolelance associated with morphine.

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