

Pharmacological Characterization of Orphanin FQ/Nociceptin and its Fragments¹

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ABSTRACT

The cloning of a fourth member of the opioid receptor family has led to the discovery of a new neuropeptide termed orphanin FQ or nociceptin (OFQ/N). Studies in CD-1 mice confirm the ability of OFQ/N to rapidly induce hyperalgesia within 15 min which is insensitive to opioid antagonists. This is followed in the next 30 min by loss of hyperalgesia and the appearance of analgesia in the tailflick assay which is readily reversed by opioid antagonists. However, the very poor affinity of OFQ/N for all the traditional opioid receptors and the insensitivity of OFQ/N analgesia to antisense oligodeoxynucleotides active against MOR-1, DOR-1 or KOR-1 sequences that selectively block μ , δ or κ_1 analgesia, respectively, make it unlikely that OFQ/N analgesia is mediated through typical opioid receptors. Blockade of the antiopioid σ system by haloperidol enhances the analgesic potency of OFQ/N of more than 100-fold. This effect is pronounced in BALB-C and Swiss-Webster

mice. Although OFQ/N alone has little analgesic activity in these mice, the blockade of σ systems with haloperidol uncovers a robust analgesic response in both strains. Two shorter OFQ/N fragments, OFQ/N(1-7) and OFQ/N(1-11), also are analgesic in CD-1 mice and their actions are reversed by the opioid antagonist diprenorphine despite very poor affinities of both peptides against [¹²⁵I]OFQ/N binding and all the opioid receptors. In antisense studies, a probe targeting the first coding exon of KOR-3 eliminates OFQ/N hyperalgesia, but not OFQ/N analgesia. Conversely, antisense probes based on the second and third coding exons are inactive against OFQ/N hyperalgesia but readily reverse κ_3 opioid analgesia. These results suggest that OFQ/N elicits both analgesia and hyperalgesia through pharmacologically distinct receptors that do not correspond to traditional opioid receptors.

Soon after the cloning of cDNA's encoding G-protein receptors selective for δ , μ and κ_1 opioids (Zimprich *et al.*, 1994; Bare *et al.*, 1994; Min *et al.*, 1994; Yasuda *et al.*, 1993; Wang *et al.*, 1994a; Reisine and Bell, 1993; Thompson *et al.*, 1993; Minami *et al.*, 1993; Chen *et al.*, 1993; Kieffer *et al.*, 1992; Evans *et al.*, 1992) we cloned a novel opioid-related receptor from mouse (KOR-3) (Uhl *et al.*, 1994; Pan *et al.*, 1994, 1995) which was homologous to clones reported by other groups (ORL₁, LC132, XOR1 and ORN7) (Fukuda *et al.*, 1994; Bunzow *et al.*, 1994; Lachowicz *et al.*, 1995; Mollereau *et al.*, 1994; Wang *et al.*, 1994b; Wick *et al.*, 1994; Uhl *et al.*, 1994; Keith *et al.*, 1994; Chen *et al.*, 1994). The mouse KOR-3 gene has been cloned (Pan *et al.*, 1996b) and alternative splicing of this gene has been observed. The initial pharmacological characterization of this receptor proved difficult. Although structurally related to the other members of the

opioid receptor family, traditional opioids have low affinity for the expressed KOR-3 clone in binding studies and functional assays, leading some to question whether it should be classified within the opioid receptor family. Several studies have documented the close relationship between the receptor encoded by the KOR-3 clone and the κ_3 receptor (Uhl *et al.*, 1994; Pan *et al.*, 1994, 1995; Brooks *et al.*, 1996; Pasternak and Standifer, 1995). The expressed KOR-3 receptor is recognized by a monoclonal antibody (mAb8D8) that blocks κ_3 binding *in vitro* and analgesia *in vivo* (Brooks *et al.*, 1996) and at least six different antisense oligodeoxynucleotides based on the second and third coding exons of KOR-3 down-regulate κ_3 analgesia in mice (Uhl *et al.*, 1994; Pan *et al.*, 1994, 1995). However, the inactivity of an additional five antisense probes targeting the first coding exon points out a major difference between KOR-3 and the κ_3 receptor, but raises the possibility that they derive from the same gene (Pasternak and Standifer, 1995; Pan *et al.*, 1995). Most investigators now agree that ORL₁/KOR-3 does not encode any of the traditional opioid receptors.

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ABBREVIATIONS: OFQ/N, orphanin FQ or nociceptin; DOR-1, a cDNA encoding a δ receptor; MOR-1, a cDNA encoding a μ receptor; KOR-1 a cDNA encoding a κ_1 receptor; KOR-3, a cDNA encoding an OFQ/N receptor; i.c.v., intracerebroventricularly; OFQ/N(1-11), Phe-Gly-Gly-Phe-Thr-Gly-Ala-Arg-Lys-Ser-Ala; OFQ/N(1-7), Phe-Gly-Gly-Phe-Thr-Gly-Ala; NalBzoH, naloxone benzoylhydrazone.

Recently, two groups reported the isolation and identification of a novel peptide from the brain with high affinity for this fourth member of the opioid receptor family. Orphanin FQ (Reinscheid *et al.*, 1995) or nociceptin (Meunier *et al.*, 1995) (OFQ/N) is a heptadecapeptide (Phe-Gly-Gly-Phe-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-Leu-Ala-Asp-Glu) which is similar structurally to dynorphin A. However, unlike the opioid peptides with their N-terminal Tyr-Gly-Gly-Phe motif, OFQ/N has a Phe-Gly-Gly-Phe sequence. OFQ/N also contains two pairs of basic amino acids, raising the possibility that the peptide may be further processed to either OFQ/N(1–11) or OFQ/N(1–7). The precursor peptide from which OFQ/N derives has been cloned from rat (Meunier *et al.*, 1995) and mouse (Houtani *et al.*, 1996; Pan *et al.*, 1996a) and the sequence reveals two additional putative peptides. One is a heptadecapeptide that is structurally similar to OFQ/N. The other is a unique peptide with some variation among species. OFQ/N binds to this fourth member of the opioid receptor family with high affinity, but is virtually inactive against the traditional opioid receptors (Reinscheid *et al.*, 1995; Meunier *et al.*, 1995).

Pharmacologically, OFQ/N has a complex series of actions. The initial studies reported that OFQ/N produces hyperalgesia, an action opposite to that typically seen with the opioid peptides (Reinscheid *et al.*, 1995; Meunier *et al.*, 1995). Among a number of other actions that have been examined, the most intriguing have been the observations that OFQ/N also can elicit analgesia (King *et al.*, 1996; Xu *et al.*, 1996; Rossi *et al.*, 1996). We now report on the pharmacology of OFQ/N and two of its fragments, OFQ/N(1–7) and OFQ/N(1–11).

Materials and Methods

OFQ/N and its fragments were synthesized by the Core Facility at MSKCC. After purification by HPLC, structures of all peptides were verified by mass spectroscopy and had peptide contents of approximately 60%. Haloperidol was purchased from Sigma (St. Louis, MO) and was administered s.c. (0.5 mg/kg). Diprenorphine, naloxone and naltrexone were gifts from the Research Technology Branch of the National Institute on Drug Abuse and were given s.c. Halothane was obtained from Halocarbon Laboratory, Hackensack, NJ.

Male CD-1 mice (24–32 g; Charles River Laboratories, Raleigh, VA) were housed in groups of five with food and water *ad libitum*. Animals were maintained on a 12-hr light/dark cycle. Peptides were administered i.c.v. under light halothane anesthesia as previously reported (Haley and McCormick, 1957). Response latencies were determined by the radiant heat tail-flick assay (D'Amour and Smith, 1941). The traditional tailflick analgesia assay used a lamp intensity which typically yielded baseline latencies between 2 to 3 sec, with a maximum cutoff score of 10 sec to minimize tissue damage. Antinociception in this paradigm was determined quantally as a doubling or greater of baseline tailflick scores, as previously reported (Rossi *et al.*, 1995; Pan *et al.*, 1995; Standifer *et al.*, 1994). For convenience, the term analgesia is used synonymously with antinociception. Dose-response curves were analyzed to generate ED₅₀ values and 95% confidence limits using the BLISS-20 program, which maximizes the log-likelihood function to fit a parallel set of Gaussian normal sigmoid curves to the dose-reponse of quantal data (Umans and Inturrisi, 1981). A second paradigm was used to look for increased sensitivity in the tailflick assay in which the lamp intensity was lowered to yield baseline latencies between 7.5 to 9.5 sec. This response has been termed hyperalgesia in previous reports (Reinscheid *et al.*, 1995; Meunier *et al.*, 1995) and for convenience we will continue to use this term. This second paradigm utilized a maximal cutoff score

of 23 sec and the response was assessed in a graded manner by comparing group means in the appropriate analysis of variance or Student's *t* tests. Repeated testing in this paradigm did not reveal any significant change in latencies over time, as indicated by saline-treated control groups tested at the same time as the experimental groups.

Antisense oligodeoxynucleotides. All phosphodiester antisense oligodeoxynucleotide sequences have already been reported in earlier studies on opioid analgesia (Pan *et al.*, 1994, 1995). They were synthesized by Midland Certified Reagent Co. (Midland, TX), purified in our laboratory and dissolved in 0.9% saline. The antisense targeting the first coding exon (GGG GCA GGA AAG AGG GAC TCC; bp 301–321), the probe based on the second coding exon (CCC AGA AGG ATG TCT GTG CCC; bp 610–630) and the antisense targeting the third coding exon (GGG CTG TGC AGA AGC CGA GA; bp 1189–1208) located between TM5-TM6 are all based on the KOR-3 clone. Because the KOR-3 gene contains an additional non-coding exon not seen in the initial cloning studies, the first coding exon corresponds to exon 2 of the KOR-3 gene (Pan *et al.*, 1996b). To facilitate comparisons with the previous antisense work on KOR-3 done before the cloning of the gene and the identification of the additional upstream noncoding exon, the terminology will be based on the original cDNA rather than the gene structure. The mismatch oligodeoxynucleotide (GGG TCG GTC AGA GAC CGA GA) is based on the antisense targeting the third coding exon and is identical in composition, differing only in the sequence of the three pairs of underlined bases. Mice were treated with the oligodeoxynucleotides (5 μ g in 2 μ l, i.c.v.) on days 1, 3 and 5 and tested with the indicated agonists on day 6, as previously described (Rossi *et al.*, 1995; Pan *et al.*, 1995; Standifer *et al.*, 1994).

Results

Hyperalgesia of OFQ/N and its fragments. Analgesia is typically assessed in the tailflick assay as a prolongation of latencies. Conversely, an increased sensitivity toward a nociceptive, or painful, stimulus results in shorter latencies. In view of the previous reports revealing OFQ/N hyperalgesia (Reinscheid *et al.*, 1995; Meunier *et al.*, 1995), we first examined the graded responses of OFQ/N in a tailflick paradigm in which the stimulus intensity had been decreased to yield a baseline latency of approximately 9 sec. By lengthening the baseline latency well beyond that typically used to examine analgesia (2–3 sec), we hoped to observe decreases in tailflick latencies more easily. In this paradigm OFQ/N rapidly lowers the tailflick latency in mice from 9.1 to 5.9 sec ($P < .0001$) (fig. 1a), confirming previous reports in the literature (Reinscheid *et al.*, 1995; Meunier *et al.*, 1995). OFQ/N hyperalgesia is dose-dependent (fig. 1b), with a maximal effect at 10 μ g. There is no significant difference between the 10- and 20- μ g doses.

OFQ/N hyperalgesia gradually resolves over time, with tailflick latencies continuing to increase to values significantly above the initial baseline values ($P < .05$), implying an analgesic action. In control studies, saline-treated mice demonstrate no significant changes in tailflick latencies over time with repeated testing (fig 1a), ensuring that the changes observed with OFQ/N over time reflect the actions of the drug rather than the effects of repeated testing.

We next examined the effects of the opioid antagonist diprenorphine. Although diprenorphine has no effect on OFQ/N hyperalgesia, it completely reverses the analgesic actions of OFQ/N (fig. 1a). The loss of the analgesic activity uncovers a persistent hyperalgesia, implying that the tailflick responses result from opposing hyperalgesic and anal-

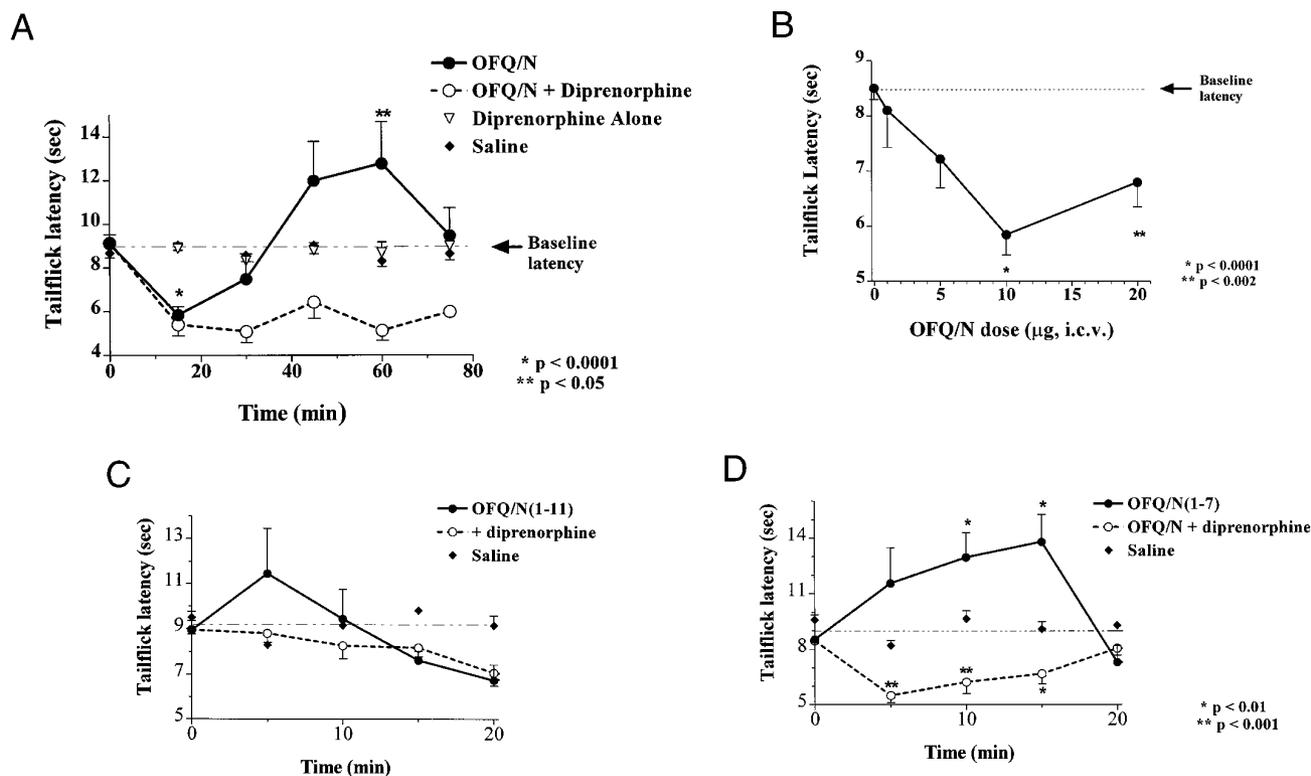


Fig. 1. Actions of OFQ/N and its fragments in a hyperalgesia assay. To facilitate the detection of hyperalgesia (*i.e.*, a decrease in the tailflick latency), this paradigm used a decreased lamp intensity that gives a longer baseline latency of approximately 9 sec. **A**, Groups of mice received OFQ/N (10 μ g, *i.c.v.*) alone (closed circles; $n = 45$), OFQ/N with diprenorphine (1 mg/kg, *s.c.*; open circles; $n = 25$), diprenorphine alone (open triangles; $n = 10$) or saline (closed diamonds; $n = 10$) and were tested at the indicated times. Repeated testing after saline or diprenorphine alone did not significantly alter latencies compared to baseline values. The 15-min OFQ/N alone point is significantly different from the saline value ($P < .0001$), as is the 60-min time point ($P < .05$). Diprenorphine significantly lowers the latencies of OFQ/N alone at 30 min ($P < .05$), 45 min ($P < .01$), 60 min ($P < .002$) and 75 min ($P < .03$). **B**, Groups of mice received the indicated doses of *i.c.v.* OFQ/N (closed circles; $n \geq 20$) and were tested after 15 min. **C** and **D**, Groups of mice ($n = 15$ – 25) received OFQ/N(1–11) (10 μ g, *i.c.v.*) or OFQ/N(1–7) (10 μ g, *i.c.v.*) alone (closed circles) or with diprenorphine (1 mg/kg, *s.c.*; open circles) and tailflick latencies were determined at the indicated times. Saline-treated animals (closed diamonds; $n = 10$) were included in each experiment. Results are the means \pm S.E.M.

gesic systems. Based on the diprenorphine sensitivity, the hyperalgesia can be classified as nonopioid although OFQ/N analgesia is opioid. Repeated testing of saline-treated mice or mice receiving only diprenorphine reveals no significant changes in baseline latency over the same testing period, confirming the validity of the assay.

The delayed response to analgesia compared to the more rapid onset of hyperalgesia raised the question of whether the analgesic actions of OFQ/N might result from its metabolism to either OFQ/N(1–11) or OFQ/N(1–7). In the hyperalgesia assay, both agents increase the tailflick latencies, consistent with an analgesic action (Fig. 1b, c). Diprenorphine eliminates these analgesic responses. Against OFQ/N(1–7), diprenorphine uncovers a hyperalgesic response similar to that seen with the parent peptide. Although diprenorphine antagonizes OFQ/N(1–11) analgesia, it does not reveal a significant hyperalgesia.

OFQ/N analgesia. We next examined OFQ/N analgesia in a traditional tailflick assay where the baseline latencies typically range between 2 to 3 sec and analgesia is defined quantally as a doubling or greater of the baseline latency (Rossi *et al.*, 1995; Pan *et al.*, 1995; Standifer *et al.*, 1994). Unlike the initial paradigm, this assay is relatively insensitive to hyperalgesia. In this traditional tailflick assay, OFQ/N is analgesic with a peak effect at 30 to 45 min, similar

to that seen in the hyperalgesia paradigm (fig. 2a). OFQ/N analgesia is dose dependent, with an apparent ceiling effect at approximately 50% and a half maximal dose of approximately 2 μ g (fig. 2b). OFQ/N analgesia remains sensitive to diprenorphine, naloxone and naltrexone, confirming the opioid nature of the action (fig. 3). To explore the possibility that OFQ/N might activate opioid systems downstream from the OFQ/N receptor, explaining the sensitivity of the response, we also examined the effects of a series of antisense probes based upon traditional opioid receptors on OFQ/N analgesia. Although all the probes that were based on either MOR-1, DOR-1 or KOR-1 block μ , δ or κ_1 analgesia, respectively, none have any effect on OFQ/N analgesia (data not shown), implying that these traditional opioid receptors are not involved.

Tonically active *sigma* systems significantly diminish the analgesic activity of opioids, particularly *kappa* agonists, and blockade of these *sigma* systems with haloperidol enhances opioid analgesia (Chien and Pasternak, 1993, 1994, 1995b; Pasternak, 1994). Although haloperidol is widely recognized as a D_2 antagonist, it has a similar, very high affinity for *sigma*₁ receptors. These actions reflect interactions between *sigma* and opioid systems because *sigma* receptor blockade alone has no analgesic effect. The possibility of a D_2 action also was ruled out by the inactivity of the potent D_2 antagonist (-)sulpiride, which does not block *sigma* receptors. In

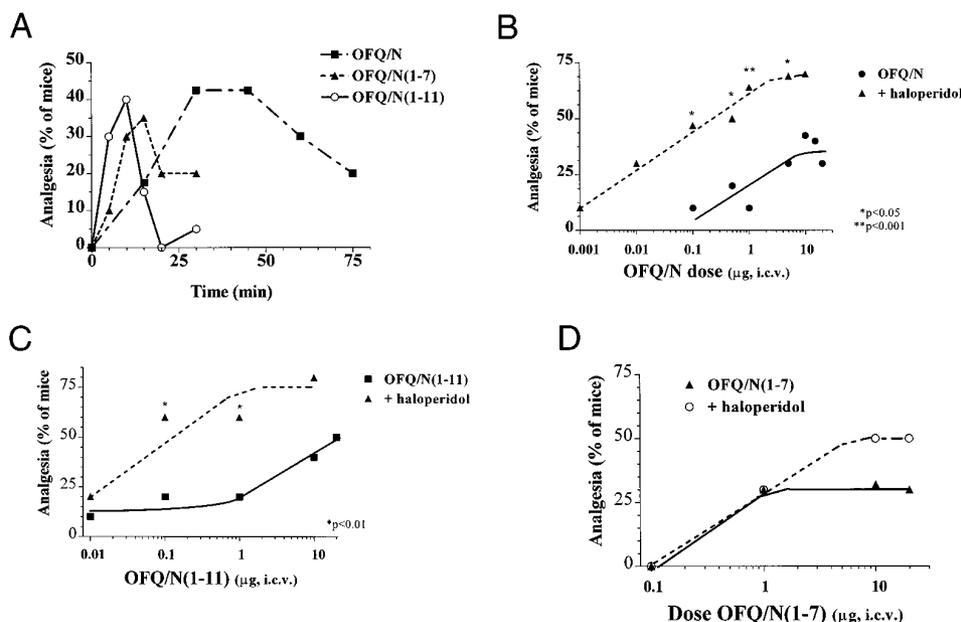


Fig. 2. Analgesic actions of OFQ/N and its fragments. The analgesic activity of OFQ/N and its two fragments were defined quantally as a doubling or greater of the baseline latency. Hyperalgesia is not readily detectable in this paradigm. All points represent at least 10 mice. A, After receiving OFQ/N (10 μg, i.c.v.), OFQ/N(1-7) (10 μg, i.c.v.) or OFQ/N(1-11) (10 μg, i.c.v.), analgesia was assessed at the indicated time. Haloperidol (0.5 mg/kg, s.c.) did not change the time to peak effect for any compound (data not shown). Dose-response curves were generated for B, OFQ/N, C) OFQ/N(1-11) or D, OFQ/N(1-7) alone or with haloperidol (0.5 mg/kg, s.c.) using peak effect data. In B the 0.1-, 0.5-, 1-, 5- and 10-μg doses with haloperidol were all significantly ($P < .03$, $P < .05$, $P < .001$, $P < .02$, $P < .03$, respectively) different from their corresponding dose alone. In C, the 0.1- and 1-μg doses with haloperidol were significantly ($P < .01$) from their corresponding doses alone. In D there were no significant differences between points on the two curves.

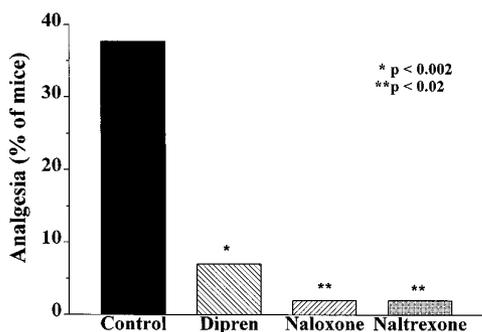


Fig. 3. Antagonism of OFQ/N analgesia by opioid antagonists. Using the traditional tailflick assay, groups of mice ($n = 45$) received OFQ/N (10 μg, i.c.v.) alone ($n = 45$) or with diprenorphine (1 mg/kg, s.c., $n = 30$), naloxone (5 mg/kg, s.c., $n = 10$) or naltrexone (5 mg/kg, s.c., $n = 10$) and analgesia assessed quantally in the standard tailflick assay. All three antagonists significantly lowered OFQ/N analgesia (diprenorphine, $P < .002$; naloxone, $P < .02$; naltrexone, $P < .02$).

time action studies, the peak analgesic effects of OFQ/N in conjunction with haloperidol are observed at the same time after injection as with OFQ/N alone (data not shown). In dose-response studies, haloperidol shifts the curve more than 300-fold to the left to a half-maximal dose of approximately 0.03 μg and increases the ceiling effect from 50% to approximately 75% (fig. 2b).

Strain differences to OFQ/N analgesia. The lack of OFQ/N analgesia in the earlier reports might be due to the strain of mouse used. Strains of mice display widely varying sensitivities toward opioid analgesics, often reflecting the tonic level of sigma activity (Chien and Pasternak, 1994; Pick *et al.*, 1991). To determine whether similar strain differences exist for OFQ/N analgesia, we compared the sensitivity of

CD-1, BALB/c and Swiss Webster mice to OFQ/N. Neither BALB/c nor Swiss Webster mice show significant analgesia with OFQ/N alone (10 μg, i.c.v.; fig. 4). Blocking the sigma system with haloperidol uncovers the analgesic sensitivity of these strains to OFQ/N. In the presence of haloperidol, OFQ/N analgesia increases significantly in both BALB/c and Swiss Webster. These observations point out the importance of the mouse strain used to examine OFQ/N analgesia and the role of sigma systems in the modulation of this activity.

OFQ/N(1-11) and OFQ/N(1-7) analgesia. In the traditional tailflick assay both OFQ/N(1-11) and OFQ/N(1-7) are analgesic, with half-maximal doses of 5 and 0.5 μg, respectively (fig. 2c, d). The onset of the response is more rapid than that seen with OFQ/N, with peak values after only 10 to 15 min (fig. 2a). Haloperidol markedly enhances OFQ/N(1-11) analgesia, shifting the analgesic dose-response curve more than 50-fold to the left to a half-maximal dose of 0.03 μg

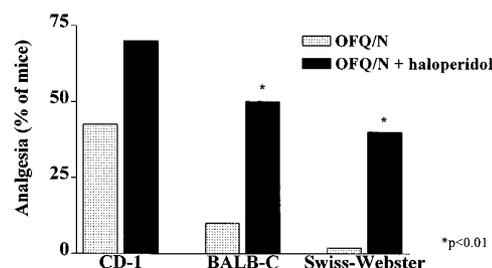


Fig. 4. Strain differences in OFQ/N analgesia. Groups of CD-1 ($n = 45$), Swiss-Webster ($n = 10$) or BALB/c ($n = 10$) received OFQ/N (10 μg, i.c.v.) alone or with haloperidol (0.5 mg/kg, s.c.; $n = 10$ for all groups) and analgesia assessed quantally in the standard tailflick assay. The analgesic response in the CD-1 mice is significantly different from BALB/c ($P < .05$) and the Swiss-Webster ($P < .009$). In the presence of haloperidol there are no significant differences among groups.

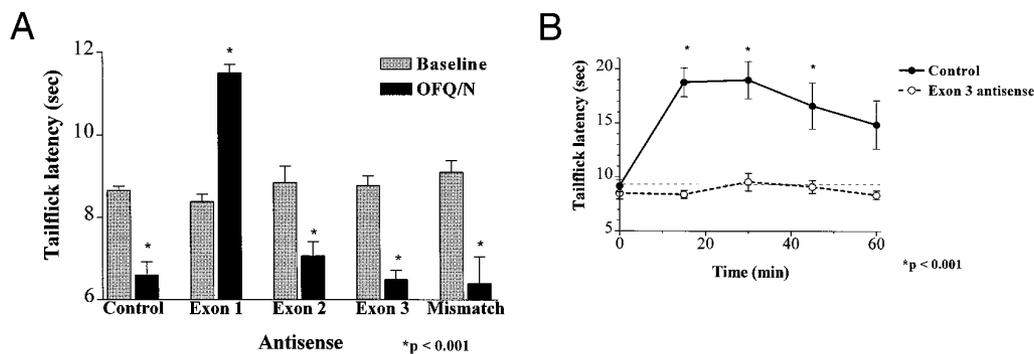


Fig. 5. Antisense effects on OFQ/N hyperalgesia. Groups of mice received antisense oligodeoxynucleotides (5 μ g, i.c.v.) targeting exon 1 ($n = 30$), exon 2 ($n = 20$) or exon 3 ($n = 30$), a mismatch probe (5 μ g, i.c.v., $n = 20$) or vehicle ($n = 30$) on Days 1, 3 and 5. A, OFQ/N (10 μ g, i.c.v.) was assessed on day 6 in the hyperalgesia paradigm with baseline latencies of approximately 9 sec. The exon 1 antisense reversed the hyperalgesic activity of OFQ/N, uncovering a significant latency increase ($P < .001$). All the other treatments did not affect the significant hyperalgesia seen in this assay. Significance was assessed by comparing the treatment group to its own control group. B, Groups of mice received either the antisense targeting exon 3 ($n = 10$) or vehicle ($n = 7$) on days 1, 3 and 5. On day 6, tailflick latencies were assessed 30 min after the κ_{3} opioid analgesic naloxone benzoylhydrazone (NalBzoH). The antisense targeting exon 3 significantly reversed κ_{3} analgesia ($P < .001$) at 15, 30 and 45 min.

along with an increase in the maximal response to approximately 75% (fig. 2c). In contrast, haloperidol has little effect on OFQ/N(1–7) analgesia (fig. 2d). The dose-response curve is not shifted and there is little change in the maximal observed response.

Antisense mapping KOR-3 in OFQ/N actions. Antisense mapping KOR-3 against κ_{3} analgesia reveals a potent blockade of analgesia by the six antisense probes targeting the second and third coding exons of KOR-3 although five probes aimed at the first coding exon are inactive (Pan *et al.*, 1994, 1995, 1996b; Pasternak and Standifer, 1995). In view of the high affinity of OFQ/N for the expressed KOR-3 receptor, we mapped KOR-3 against OFQ/N hyperalgesia. Although inactive against κ_{3} analgesia (Pasternak and Standifer, 1995; Pan *et al.*, 1995), the antisense based on the first coding exon of KOR-3 (Pan *et al.*, 1996b), effectively blocks OFQ/N hyperalgesia, uncovering a significant analgesic response (fig. 5a). The specificity of this effect is confirmed by the persistent hyperalgesia observed in vehicle or mismatch-treated mice. The antisense probes targeting the second and third coding exons of KOR-3, do not affect OFQ/N hyperalgesia (fig. 5a) despite their ability to block κ_{3} analgesia in traditional tailflick assay (Pasternak and Standifer, 1995; Pan *et al.*, 1995). The activity of the antisense targeting exon 3 against κ_{3} analgesia is not limited to the traditional tailflick assay. In the hyperalgesia

testing paradigm, this antisense oligodeoxynucleotide still blocks naloxone benzoylhydrazone (NalBzoH) analgesia (fig. 5b) despite its inactivity against OFQ/N hyperalgesia. Thus, the inability of this antisense to block hyperalgesia cannot be explained by technical factors associated with the antisense approach.

Discussion

Originally discovered as a ligand for the orphan opioid receptor clone (Meunier *et al.*, 1995; Reinscheid *et al.*, 1995), OFQ/N is interesting from a number of perspectives (table 1). Initial studies indicated that OFQ/N induces hyperalgesia based on its ability to lower latencies in modified antinociceptive assays, an effect opposite that of traditional opioid analgesics. Based on these reports, we initially examined OFQ/N actions using a testing paradigm with longer baseline latencies to enhance our ability to observe hyperalgesia. Traditional tailflick assays with their short baseline latencies are often insensitive to hyperalgesia. Our studies replicate the hyperalgesia previously reported (Reinscheid *et al.*, 1995; Meunier *et al.*, 1995). OFQ/N significantly shortens the tailflick latencies. The ability to observe this action may be dependent on the species and strains tested, as well as experimental paradigms, possibly explaining the difficulty some groups have experienced trying to demonstrate this

TABLE 1
Pharmacological profile of OFQ/N actions

| Agent and Behavior | Antisense | | | Opioid Antagonists | KOR-3 Binding (K_i) | Opioid Binding (K_i) |
|------------------------|-----------|-----------|-----------|--------------------|-------------------------|--------------------------|
| | Exon 1 | Exon 2 | Exon 3 | | | |
| OFQ/N | | | | | 0.1nM | >500nM |
| Hyperalgesia | Blocks | No effect | No effect | No effect | | |
| Analgesia | No effect | Blocks | Blocks | Block | | |
| OFQ/N(1–11) | | | | | 55 nM | >1000nM |
| Analgesia | | | | Block | | |
| OFQ/N(1–7) | | | | | >1000 nM | >1000nM |
| Analgesia | | | | Block | | |
| NalBzoH (κ_3) | | | | | 310 nM | <10nM |
| Analgesia | No effect | Blocks | Blocks | Block | | |

The antisense and opioid antagonist data are summarized from data presented herein. The binding data are from the literature (Mathis *et al.*, 1997; Pan *et al.*, 1996; King *et al.*, 1987). The affinity of the agents for opioid sites includes total μ , δ , κ_{1} and κ_{3} . NalBzoH labels the opioid receptors with affinities less than 1 nM, with the exception of δ binding, which is slightly higher.

response. For example, we see hyperalgesia in mice after supraspinal administration, but not with spinal administration (King *et al.*, 1997) and we have not seen it in rats (M. King and G. W. Pasternak, unpublished observations). Although initially reported as hyperalgesia (Reinscheid *et al.*, 1995; Meunier *et al.*, 1995), the underlying mechanisms are not clear and more detailed evaluations are needed to discern the mechanisms responsible for this effect.

OFQ/N functionally reverses the analgesic actions of a number of opioids (Mogil *et al.*, 1996b) and, in one study, produced an action suggesting hyperalgesia that actually reflected the antagonism of stress-induced opioid analgesia (Mogil *et al.*, 1996a). However, this mechanism may be dependent on the paradigms used and does not appear to explain the decrease in tailflick latencies in our own studies. Unlike the other report, we do not observe an opioid stress-induced analgesia in our studies. Diprenorphine alone has no significant effect upon tailflick latencies, even with repeated testing. This would appear to eliminate any significant stress-induced opioid activity. Thus, it is unlikely that decreased tailflick latencies induced by OFQ/N is mediated by the reversal of opioid systems in our studies. However, not all stress-induced analgesia is reversed by opioid antagonists, implying a nonopioid component as well (Spiaggia *et al.*, 1979), and it is still possible that OFQ/N is counteracting this system.

The increased latencies seen in the hyperalgesia paradigm were unexpected, particularly their sensitivity toward opioid antagonists. Thus, OFQ/N actions in the hyperalgesia paradigm appear to result from the summation of two opposing actions. Although the hyperalgesia is relatively short-lasting after OFQ/N alone, blockade of the analgesia by diprenorphine uncovers a persistent hyperalgesia which extends for more than 75 min, the longest time examined. The concept of two opposing actions also is supported by the antisense studies. Down-regulating the first coding exon of ORL-1/KOR-3 with antisense A eliminates OFQ/N hyperalgesia and immediately uncovers an underlying analgesic component of OFQ/N activity.

OFQ/N analgesia was not observed in initial reports (Reinscheid *et al.*, 1995; Meunier *et al.*, 1995), possibly due to species and strain differences. The demonstration of OFQ/N analgesia is dependent on the strain of mouse examined. CD-1 mice have proven sensitive to a wide variety of analgesics, including opioids inactive in other strains. Thus, the sensitivity of these mice to OFQ/N is consistent with previous observations with opioids.

Having observed the increased latencies in the hyperalgesia paradigm, we reexamined OFQ/N actions in a traditional tailflick assay. OFQ/N is analgesic in these studies, with a well defined dose-response curve. Ceiling effects made it difficult to accurately define the analgesia from OFQ/N alone. This problem was overcome by including haloperidol to block the sigma system. The well established ability of sigma receptors to modulate opioid analgesia (Chien and Pasternak, 1995a, b; Pasternak, 1994; Chien and Pasternak, 1993) extends to OFQ/N analgesia as well. Haloperidol enhances OFQ/N efficacy and dramatically increases the analgesic potency of OFQ/N over 300-fold. Sigma receptors also play a role in OFQ/N analgesia in BALB-C and Swiss Webster mice where haloperidol uncovers a robust OFQ/N analgesia. Clearly, the strain of mouse has a major influence upon the

observed OFQ/N pharmacology due to the tonic activity of sigma systems.

OFQ/N analgesia is readily reversed by opioid antagonists. This was particularly surprising in view of the very poor affinity of the opioids for the expressed ORL₁/KOR-3 receptor. Although it is possible that the opioid sensitivity of OFQ/N analgesia reflects the activation of opioid pathways downstream from the OFQ/N binding site, this seems unlikely based on antisense studies. Antisense probes which effectively block either *mu*, *delta*, or *kappa*₁ analgesia (Pasternak and Standifer, 1995; Rossi *et al.*, 1995; Standifer *et al.*, 1994) have no effect against OFQ/N analgesia. If OFQ/N were releasing endogenous opioids, we would have expected one of the antisense probes targeting the traditional opioid receptors to block OFQ/N analgesia.

The analgesic actions of OFQ/N(1–11) and OFQ/N(1–7) are interesting from several perspectives. OFQ/N(1–11) and OFQ/N(1–7) display reasonable analgesic actions despite poor affinities against ¹²⁵I[Tyr¹⁴]OFQ/N binding in KOR-3 transfected cell lines (K_i 55 nM and > 1 μ M, respectively) compared to OFQ/N (K_i 0.09 nM). As with OFQ/N, OFQ/N(1–11) and OFQ/N(1–7) analgesia is easily antagonized by the opioid antagonist diprenorphine, despite the very poor affinity of either peptide for the traditional opioid receptors (K_i > 1 μ M) (Mathis *et al.*, 1997). The two shorter OFQ/N peptides differ from each other. Like OFQ/N, OFQ/N(1–11) analgesia is dramatically potentiated by haloperidol although OFQ/N(1–7) analgesia is relatively unaffected. The slower onset of OFQ/N analgesia compared to the two smaller fragments (fig. 2a) also is interesting, although the reasons remain unclear. The delay in the appearance of OFQ/N analgesia may be due to the opposing hyperalgesia seen at early times. Alternatively, OFQ/N analgesia may be due to its conversion to an active metabolite. In preliminary studies using [¹³¹I][Tyr¹⁴]OFQ/N administered intracerebroventricularly in mice, more than 75% of the peptide is metabolized within 15 min (J. P. Mathis and G. W. Pasternak, unpublished observations).

The ability to discriminate between OFQ/N hyperalgesia and analgesia in a number of pharmacological paradigms strongly implies distinct receptor mechanisms. OFQ/N hyperalgesia is insensitive to opioid antagonists although analgesia is readily reversed by naloxone, naltrexone and diprenorphine. While the possibility that OFQ/N analgesia activates downstream opioid systems that are responsible for the sensitivity to opioid antagonists cannot be excluded, the failure of antisense probes targeting MOR-1, DOR-1 or KOR-1 to block OFQ/N analgesia makes this less likely. Antisense mapping also implies distinct receptors. The antisense that targets the first coding exon of KOR-3 blocks OFQ/N hyperalgesia without reversing OFQ/N analgesia, much like its inactivity against *kappa*₃ analgesia (Pasternak and Standifer, 1995; Pan *et al.*, 1995). At the same time, hyperalgesia remains untouched by the two antisense oligodeoxynucleotides aimed against the second and third coding exons despite their ability to block *kappa*₃ analgesia (Pasternak and Standifer, 1995; Pan *et al.*, 1995). Thus, all the antisense probes are active in at least one assay, ruling out technical problems, such as diffusion, stability or secondary mRNA structure.

Biochemical studies are consistent with OFQ/N receptor heterogeneity (Mathis *et al.*, 1997). Functionally, both

OFQ/N and OFQ/N(1–11) inhibit forskolin-stimulated cAMP accumulation in mouse brain by 40 to 50% (IC_{50} values < 10 nM). The potency of OFQ/N(1–11) in these cyclase assays contrasts with its relatively poor affinity in ^{125}I [Tyr¹⁴]OFQ/N binding studies in KOR-3 transfected Chinese hamster cells (Pan *et al.*, 1996c). As with OFQ/N analgesia, opioid antagonists effectively reverse the inhibition of forskolin-stimulated cyclase by OFQ/N and OFQ/N(1–11) (Mathis *et al.*, 1997). ^{125}I [Tyr¹⁴]OFQ/N binding studies in brain also suggest heterogeneity (Mathis *et al.*, 1997). Binding in transfected cell lines is consistent with a single site (K_D 0.1 nM) (Pan *et al.*, 1996c; Reinscheid *et al.*, 1995). However, in mouse brain homogenates competition studies reveal shallow Hill slopes for a number of compounds and saturation studies with ^{125}I [Tyr¹⁴]OFQ/N reveal a curvilinear Scatchard plots. Analysis of these saturation studies reveals a very high affinity site (K_D 4 pM) not seen in transfected cell lines (K_D 40 pM) (Pan *et al.*, 1996c), as well as a more abundant lower affinity site (K_D 0.9 nM) similar to that seen in rats using 3H -OFQ/N (5 nM) (Dooley and Houghten, 1996). Thus, radiolabeled OFQ/N binding to brain membranes differs from that seen in transfected cell lines and may indicate binding site heterogeneity.

In conclusion, OFQ/N is a complex and intriguing peptide. Although OFQ/N is hyperalgesic, it also can elicit analgesia that is readily antagonized by opioid antagonists. Different receptors appear to mediate OFQ/N analgesia and hyperalgesia and additional studies will be needed to define them. These studies raise the possibility that OFQ/N may be one of a family of pharmacologically relevant neuropeptides acting through multiple OFQ/N receptors.

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