

## **Endogenous orphanin FQ / nociceptin is involved in the development of morphine tolerance**

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OFQ/N, orphanin FQ/nociceptin; MOP receptor,  $\mu$ -opioid receptor; NOP receptor, N/OFQ peptide receptor; ORL-1 receptor, opioid receptor-like 1 receptor; NMDA, N-methyl-D-aspartate; AMPA, alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid

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## Abstract

The neuropeptide orphanin FQ/nociceptin (OFQ/N) has been shown to counteract several effects of endogenous and exogenous opioids and it has been proposed as an opioid-modulating agent involved in the development of morphine tolerance and dependence. However, conflicting results have been obtained from animal models using different protocols to induce morphine tolerance. Here we report that both genetic and pharmacological blockade of OFQ/N signaling can effectively prevent development of morphine tolerance. OFQ/N knockout mice injected daily with low doses of morphine (10 mg/kg) fail to develop tolerance even after 3 weeks of treatment while their wildtype littermates show profound tolerance starting after 10 days. Similarly, co-administration of morphine together with the synthetic NOP antagonist, J-113397, is able to block tolerance development in normal mice. These data indicate that release of endogenous OFQ/N following morphine administration might produce a gradual decline of analgesic potency, i.e. tolerance. Interestingly, tolerant and non-tolerant groups of mice receiving repeated daily low morphine doses did not differ in their withdrawal behavior after naloxone injection. In contrast, mice receiving escalating doses of morphine developed analgesic tolerance independent of their OFQ/N genotype whereas withdrawal symptoms were attenuated in OFQ/N deficient animals. These results indicate that the endogenous OFQ/N system is differentially involved in morphine tolerance development and establishment of opiate dependence, depending on the specific morphine dosage regimen. Furthermore, it suggests that OFQ/N antagonists could provide a novel therapeutic strategy to attenuate morphine tolerance development.

## Introduction

Morphine and related opioids are still the most powerful and widely used drugs in the clinical management of severe pain. Long-term use of opioids is however limited by the development of tolerance and dependence. Tolerance is a gradual loss in drug effect upon repeated administration, requiring increasing doses to maintain analgesia. Dependence reflects a change in neuronal homeostasis that results in withdrawal symptoms upon cessation of drug administration. Studies on knockout mice have shown that activation of  $\mu$ -opioid receptors (MOP) is responsible for morphine-induced analgesia, tolerance and dependence (Matthes et al., 1996). However, the mechanisms of adaptive changes downstream of MOP activation are still poorly understood. Therefore, elucidating the molecular and neurobiological mechanisms of opioid tolerance has been recently compared to the “search for the holy grail” in opioid research (Kieffer and Evans, 2002).

The search for physiological correlates of opioid tolerance has indicated a number of protein kinases, ion channels, second messenger-synthesizing enzymes, glutamate receptors, cytoskeletal proteins and neurotrophic factors to be involved (Nestler, 1997; Williams et al., 2001). Also, the inability of various opioid drugs to trigger internalization of  $M\mu$  receptors has been linked to their potential to produce tolerance (Whistler et al., 1999). At the level of neuronal circuits, the induction of so-called “anti-opioid” or “opioid-modulating systems” has been proposed for a long time (Gillman and Lichtigfeld, 1981; Rothman, 1992). The model implies that repeated morphine administration produces enhanced activity in opioid-modulating systems that in turn

attenuate the analgesic opioid effect and gradually lead to tolerance. Several neurotransmitters have been identified to play such an opioid-modulating role, e.g. cholecystokinin, neuropeptide FF and the dynorphin opioid peptides (Mollereau et al., 2005), while their role in opioid dependence is still discussed controversially.

More recently, the fourth member of the opioid peptide family, orphanin FQ / nociceptin (OFQ/N) (Meunier et al., 1995; Reinscheid et al., 1995), was reported to counteract several opioid-mediated effects. OFQ/N binds selectively to the NOP receptor (previously known as ORL-1; Reinscheid et al., 1996) and induces intracellular effects similar to opioids, such as hyperpolarization (Connor et al., 1996), inhibition of voltage-gated  $\text{Ca}^{2+}$ -channels (Knoflach et al., 1996) or inhibition of adenylate cyclase (Reinscheid et al., 1995). However, OFQ/N and NOP are not co-localized with the classical opioid peptides or receptors (Neal et al., 1999). Central administration of OFQ/N can attenuate morphine- and stress-induced analgesia (Mogil et al., 1996a; Mogil et al., 1996b). These studies indicated an opioid-modulating role for OFQ/N, however they did not demonstrate activation of the endogenous OFQ/N system following opioid receptor stimulation. Further studies using central injection of OFQ/N antisera showed attenuated development of tolerance to morphine- and electroacupuncture-induced analgesia (Tian and Han, 2000). Most importantly, chronic morphine administration was found to increase OFQ/N peptide levels in brain after 3 – 5 days of morphine treatment, coinciding with the onset of analgesic tolerance (Yuan et al., 1999).

Studies with knockout mice were less consistent. While one study showed that NOP receptor-deficient mice develop less tolerance to morphine analgesia than their wildtype littermates (Ueda et al., 2000), others found no effects in either NOP- (Mamiya et al., 2001) or OFQ/N-deficient animals (Kest et al., 2001). The latter studies used accelerated morphine dosage regimen that are known to produce higher degrees of tolerance than the once daily administration of the former study. Co-administration of morphine with synthetic NOP antagonists (Ueda et al., 2000; Zaratin et al., 2004) significantly attenuated morphine tolerance in normal mice although treatment schedules were relatively short (up to 6 days) and did not address temporal or dosage effects which would be more reminiscent of clinical situations. Together, these studies indicate a role of OFQ/N in the modulation of morphine tolerance but raise questions whether different degrees of morphine tolerance involve differential modulation by OFQ/N. In addition, conflicting results from the knockout studies indicate differences either in genetic background, developmental compensation, or experimental methodology that demand further attention.

In the present study we investigated the role of OFQ/N in morphine tolerance using both genetic and pharmacological approaches of attenuated OFQ/N signaling. In contrast to previous studies we studied development of morphine tolerance over three weeks and at different dosage regimen. We provide evidence that OFQ/N differentially affects analgesic tolerance depending on the morphine dose used and that pharmacological blockade of NOP receptors is able to prevent development of tolerance for a prolonged time. These data implicate the OFQ/N system as a valuable candidate for development of

drugs that might prevent development of morphine tolerance during treatment of severe pain.

## Methods

**Animals.** Male C57BL/6N mice (National Cancer Institute, Bethesda, MD) or OFQ/N knockout mice (age 8-14 weeks, weight 25-30g) were used for all experiments. The prepro-OFQ/N knockout animals and wildtype littermates used in this study were progeny of hybrid C57BL/6J x 129/Ola mice that were described previously (Köster et al., 1999). All animals were group-housed (3-5 animals per cage) under controlled conditions (temperature  $21^{\circ}\text{C} \pm 2$ ; relative humidity 50-60 %; 12-h light-dark cycle, lights on 6:00 AM) with free access to food and water. All animal experiments were approved by an institutional review board for animal welfare (IACUC).

**Drugs.** Morphine sulfate and naloxone hydrochloride were obtained from Sigma (St. Louis, MO). The NOP antagonist J-113397 (1-[(3R,4R)-1-cyclooctylmethyl-3-hydroxymethyl-4-piperidyl]-3-ethyl-1, 3-dihydro-2H-benzimidazol-2-one) was prepared as described before (De Risi et al., 2001). All compounds were dissolved in sterile 0.9 % saline and administered by intraperitoneal (i.p.) injection in a total volume of 100  $\mu\text{l}$  per animal.

**Acute analgesic effect of different doses of morphine in OFQ/N<sup>+/+</sup> and OFQ/N<sup>-/-</sup> mice.** OFQ/N<sup>+/+</sup> and OFQ/N<sup>-/-</sup> mice were i.p. injected with various doses of morphine (0, 5, 10, and 20 mg/kg). Analgesia was measured 30 minutes after morphine injection. Response to nociceptive stimuli was measured by tail-flick test using an electronically controlled tail-flick analgesiameter (Ugo Basile, Comerio-Varese, Italy) equipped with a 20 W infrared lamp as the heat source. In all tests, the cutoff time was fixed to 22 s to prevent tissue damage. Reaction time until the mouse removed its tail from the heat source was monitored. Mean baseline latency was measured for each animal before morphine administration

**Development of morphine tolerance in OFQ/N<sup>+/+</sup> and OFQ/N<sup>-/-</sup> mice by daily low doses of morphine.** Morphine (10 mg/kg, i.p.) was administered to both groups of animals and analgesia was measured 30 minutes following morphine injection. Both groups were injected with morphine daily for 22 days and analgesia was measured every 3 days to monitor the temporal pattern of tolerance development but also avoid habituation to the procedure. Basal pain perception was assessed the day before the first morphine injection. One group of OFQ/N<sup>-/-</sup> mice was injected daily with saline to control for potential stress effects.

**Development of morphine tolerance in OFQ/N<sup>+/+</sup> and OFQ/N<sup>-/-</sup> mice by escalating doses of morphine.** Escalating doses of morphine were administered by i.p. injection at an interval of 12 hours over a period of 7 days. The morphine dose was increased as follows: 2 x 10 mg/kg (Day 1 and 2), 2 x 20 mg/kg (Day 3 and 4) and 2 x 40 mg/kg (Day



5, 6 and 7). Such a protocol has been shown to produce robust morphine tolerance and dependence (Fernandes et al., 1977; Kest et al., 2002). Basal pain perception was measured the day before the first morphine injection and analgesia was measured 30 minutes after morphine injection on day 1, 4, 6 and 7.

**Effect of J-113397 on the development of morphine tolerance by daily low doses of morphine.** NOP antagonist J-113397 (20 mg/kg, i.p.) or saline was administered to each group of C57BL/6N mice 10 minutes before morphine or saline injection. Morphine (10 mg/kg, i.p.) or saline was administered to the respective groups and analgesia was measured as tail-flick latency 30 minutes after morphine or saline injection, respectively. The four treatment groups were a) J-113397/morphine, b) saline/morphine, c) J-113397/saline and d) saline/saline. Basal pain perception was assessed the day before the first morphine injection. Treatment was once per day for 22 days. To avoid habituation to the procedure, pain perception was only measured every 3 days.

**Analysis of naloxone-induced withdrawal symptoms.** Morphine-tolerant mice were injected with naloxone (1 mg/kg, i.p.) 3 hours after the last morphine administration of the treatment schedule. Immediately following naloxone administration, mice were placed individually into test chambers (30 x 19 x 12 cm). Signs of naloxone-precipitated withdrawal were recorded for 15 min. Escape jumping attempts were most prominent among all symptoms and the number of jumps was counted as a quantitative measure of morphine withdrawal behavior.

**Statistical analysis.** Data are presented as mean values  $\pm$  S.E.M. Statistical significance between treatment groups was established by analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test. Individual groups were compared by Student's *t* test. A level of  $p < 0.05$  was considered significant.

## Results

The acute analgesic effect of increasing doses of morphine was investigated in OFQ/N<sup>+/+</sup> and OFQ/N<sup>-/-</sup> mice. Both genotypes showed dose-dependent analgesic responses (Fig. 1) and there was no significant difference between genotypes, implying that OFQ/N is not involved in the acute analgesic effect produced by morphine. Both groups reached maximal analgesic latency at a dose of 10 mg/kg morphine and this dose was therefore chosen for subsequent experiments analyzing analgesic tolerance.

OFQ/N<sup>+/+</sup> and OFQ/N<sup>-/-</sup> mice received once-daily low doses of morphine (10 mg/kg, i.p.) for 22 days to induce tolerance. OFQ/N<sup>+/+</sup> mice started to develop morphine tolerance after 7 days of daily morphine injection and tail flick latency returned to baseline level by day 22, indicating that morphine had completely lost its analgesic effect due to tolerance development. In contrast, development of morphine tolerance was significantly attenuated in OFQ/N<sup>-/-</sup> mice over the 3-week course of the experiment (Fig. 2). OFQ/N knockout mice showed significant morphine-induced analgesia throughout the treatment and their analgesic responses were significantly different from OFQ/N<sup>+/+</sup> mice on days

16, 19 and 22 ( $p < 0.001$ ). Analgesic thresholds were not affected by daily saline injections in OFQ/N<sup>-/-</sup> mice, indicating that repeated stress produced by the daily injections did not change the animals' nociceptive responses.

Many previous studies used escalating doses of morphine to induce and study tolerance. Therefore, OFQ/N<sup>+/+</sup> and OFQ/N<sup>-/-</sup> mice were injected with escalating doses of morphine to induce rapid development of morphine tolerance (Fig. 3). Both groups of mice developed morphine tolerance and their tail flick latencies reached basal levels after 7 days of morphine administration. Escalating morphine doses produced faster development of morphine tolerance than low constant doses of morphine. Mice started to develop tolerance on the 4<sup>th</sup> day of morphine injection and were completely tolerant after 7 days. These results indicate that administration of escalating morphine doses induces a different type of analgesic tolerance that is not susceptible to OFQ/N modulation.

Since our previous experiments showed that repeated low doses of morphine, but not escalating doses, could produce a form of analgesic tolerance that might be modulated by OFQ/N, we next investigated whether release of endogenous OFQ/N was necessary for this adaptive change in nociceptive responsiveness. Using C57BL/6N mice and a repeated low-dose morphine regimen (10 mg/kg) we co-injected one group of animals daily with the NOP antagonist, J-113397 (20 mg/kg, i.p.), while the other group of mice received saline injections. Both groups of mice were treated for 22 days. As illustrated in Fig. 4, both groups of animals displayed maximal analgesic effects between day 1 and day 7 of treatment. After 10 days of treatment the saline/morphine group showed gradual

development of tolerance that increased to complete analgesic tolerance after 19 days of treatment. In contrast, mice injected with low constant doses of morphine together with the NOP receptor antagonist J-113397 failed to develop tolerance even after 22 days of morphine treatment. Analgesia in the J-113397/morphine treated animals was significantly different from the saline/morphine group after day 10 and significance increased thereafter ( $p < 0.001$ ). Daily injections of J-113397 alone did not produce any analgesic effects (Fig. 4) and thus the analgesia observed after 10 days in the J-113397/morphine treated group can be solely attributed to a persistent anti-nociceptive effect of morphine. Daily injections of saline for 22 days also did not affect analgesic thresholds. These data show that pharmacological blockade of OFQ/N signaling can prevent development of morphine tolerance even after prolonged morphine administration.

Chronic exposure to morphine produces dependence that becomes apparent after cessation of morphine administration. Withdrawal was precipitated with naloxone in all groups of morphine-tolerant mice following the last morphine administration and the number of escape jumps was used as a quantitative measure of withdrawal behavior. C57BL/6N mice receiving repeated low doses of morphine with or without the NOP antagonist J-113397 showed no difference in the number of naloxone-precipitated jumps (Fig. 5A). Also, OFQ/N<sup>+/+</sup> and OFQ/N<sup>-/-</sup> mice treated with daily low doses of morphine over 22 days displayed similar numbers of jumps (Fig. 5B). However, OFQ/N<sup>+/+</sup> mice made tolerant by escalating doses of morphine showed increased numbers of withdrawal jumps, albeit failing to reach significance when compared to OFQ/N<sup>-/-</sup> mice under the

same treatment ( $p = 0.2$ , unpaired  $t$  test; Fig. 5C). On average, OFQ/N<sup>+/+</sup> mice displayed twice more escape jumps as compared to the other groups of mice who received constant doses of morphine. Withdrawal jumping in OFQ/N<sup>-/-</sup> mice treated with escalating doses of morphine was at the same level as in animals made tolerant using daily low doses of morphine. Neither C57BL/6N mice treated daily with J-113397 alone or saline nor OFQ/N<sup>-/-</sup> mice injected with saline for 22 days displayed any withdrawal jumping after naloxone injection (data not shown). These results indicate that OFQ/N signaling enhances expression of withdrawal signs when dependence is established by escalating doses of morphine. In contrast, the endogenous OFQ/N system does not appear to contribute to the development of morphine dependence when morphine is administered daily at low doses.

## Discussion

In the present study we sought to investigate the role of OFQ/N in morphine tolerance and dependence. We provide evidence from genetic and pharmacological models that release of endogenous OFQ/N might be involved in the development of analgesic tolerance to morphine, if morphine is administered repeatedly at low doses. OFQ/N signaling does not appear to influence the acute analgesic effect of morphine since dose-responses of acutely administered morphine were independent of OFQ/N genotype. We also observed that higher doses of morphine, administered in a regimen that induces rapid tolerance, produce a fundamentally different type of analgesic tolerance and opioid

dependence. This factor has often been ignored in previous studies and could account for some of the conflicting results reported earlier about the role of OFQ/N in opioid tolerance and dependence. Our results demonstrate that analgesic tolerance induced by low doses of morphine is susceptible to OFQ/N modulation while tolerance produced by escalating doses of morphine is independent of OFQ/N. On the other hand, the degree of opioid dependence, when measured as precipitated withdrawal behavior, is not modulated by the OFQ/N system when dependence is induced by low doses of morphine. In contrast, expression of withdrawal symptoms in animals made tolerant by escalating morphine doses appears to involve the OFQ/N system. These results indicate that even though the primary readout of analgesic tolerance (i.e. lack of morphine-induced analgesia) might be identical in the two tolerance models, the underlying neurobiological mechanisms might be different at the systems level.

Our studies confirm and extend earlier results by Ueda et al. using NOP receptor-deficient mice and a protocol of inducing morphine tolerance using low doses (10 mg/kg) over 6 days (Ueda et al., 2000). They found that  $NOP^{-/-}$  mice showed attenuated development of morphine tolerance as compared to their wildtype littermates. While their treatment schedule was not long enough to induce complete analgesic tolerance (wildtype animals still showed residual morphine-induced analgesia after 6 days of treatment), the study indicated that absence of NOP receptors attenuated development of analgesic tolerance to low doses of morphine. In addition, they showed that pharmacological blockade of OFQ/N signaling by the antagonist J-113397 is similarly efficient in preventing development of tolerance to the same low doses of morphine over a course of

6 days. Our present study extends these results by showing that analgesic tolerance to morphine does not develop in mice lacking the OFQ/N peptide and can be attenuated in normal mice for up to three weeks by co-administration of low morphine doses together with J-113397. Unfortunately, the study by Ueda et al. did not report naloxone-precipitated withdrawal behavior in the groups of mice made tolerant with low doses of morphine. However, they showed that when using escalating doses of morphine over 4 days (up to 100 mg/kg/animal), NOP receptor-deficient mice displayed reduced signs of jumping behavior as a measure of withdrawal, which complements our findings.

On the other hand, the previous studies by Kest et al. used three-times daily injections of escalating doses of morphine over 3 days (10, 20 and 40 mg/kg on day 1, 2 and 3, respectively) and failed to detect differences in analgesic tolerance between prepro-OFQ/N knockout mice and wildtype littermates (Kest et al., 2001). Also, the study by Mamiya et al. used twice daily administrations of 10 mg/kg morphine over 5 days in NOP receptor-deficient mice and found no difference in analgesic tolerance when comparing them to wildtype animals (Mamiya et al., 2001). Our present studies show that these elevated doses of morphine produce OFQ/N-insensitive analgesic tolerance. Mamiya et al. also found that withdrawal behavior in NOP receptor knockout mice is attenuated if these animals were made tolerant by higher doses of morphine. With respect to morphine withdrawal, Kest et al. used a protocol of continuous morphine administration (morphine pellet implantation for 72 h) and found that prepro-OFQ/N knockout mice displayed increased jumping behavior after precipitation of withdrawal by naloxone. The latter observation indicates that continuous administration of morphine

might produce a qualitatively different type of tolerance and dependence that might be modulated yet differently by OFQ/N. Clearly, further studies in this direction are necessary to characterize the different types of analgesic tolerance induced by various morphine administration protocols and the involvement of opioid-modulating systems. In the present studies analgesic tolerance was assessed by tail-flick which measures a spinal nociceptive reflex. There is generally a good correlation between nociceptive tests measuring spinal and supra-spinal mechanisms of analgesia and morphine tolerance. Further studies might be necessary to confirm that blockade of OFQ/N signaling also affects morphine tolerance in tests of supra-spinal analgesia, e.g. the hot plate assay, although current models indicate that analgesic tolerance to repeated morphine administration involves central mechanisms (Maldonado et al., 1996).

It should be noted that the pharmacological selectivity of the synthetic NOP antagonist J-113397 has been questioned recently as it was found to produce rewarding effects in NOP receptor knockout mice (Koizumi et al., 2004). However, there are numerous studies demonstrating in vitro and in vivo activity of J-113397 at NOP receptors with high selectivity and potency. Notably, OFQ/N-mediated effects were found to be reversible by J-113397 administration, for example OFQ/N-induced hyperalgesia (Ozaki et al., 2000), sensitizing effects of OFQ/N on kainate-induced seizures (Bregola et al., 2002), or anxiolytic effects of OFQ/N agonists (Varty et al., 2005). Importantly, no effects of J-113397 on basal pain perception have been reported (Ozaki et al., 2000) and our present data confirm these results.



We have reported previously that OFQ/N-deficient mice fail to adapt to repeated stress (Köster et al., 1999). Daily saline injections in OFQ/N<sup>-/-</sup> mice failed to affect their nociceptive threshold, demonstrating that impaired habituation to repeated stress or excessive stress-induced analgesia can not be responsible for attenuated morphine tolerance in OFQ/N<sup>-/-</sup> mice, but it correlates with the absence of OFQ/N signaling. It should be noted that distinct biological activities have been described for other prepro-OFQ/N processing products. The knockout mice used in this study do not express any part of prepro-OFQ, including nocistatin and prepro-OFQ/N 160-187 (also termed OFQ II/Noc II). Both peptides do not act on the NOP receptor which is highly selective for its endogenous ligand OFQ/N (Reinscheid et al., 1996) and receptor-mediated mechanisms for nocistatin or prepro-OFQ 160-187 still remain to be discovered (Neal et al., 2003). A number of divergent phenotypes have been described for NOP receptor-deficient mice and OFQ/N knockout mice, respectively. While NOP<sup>-/-</sup> mice display normal emotional behaviors, including stress responses (Mamiya et al., 1998), they were found to show improved learning and memory (Manabe et al., 1998). Conversely, OFQ/N<sup>-/-</sup> mice show impaired stress responses with increased anxiety-like behavior and inability to adapt to repeated stress (Köster et al., 1999). The neurobiological basis for these discrepancies is currently unknown but certainly rewards further attention. Both NOP<sup>-/-</sup> and OFQ/N<sup>-/-</sup> mice display attenuated development of tolerance to the analgesic effects of morphine when using repeated administration of low morphine doses (Ueda et al., 2000; this study). Thus, converging evidence from studies using NOP receptor antagonists and NOP receptor knockout mice, together with the present findings in OFQ/N knockout animals indicate that OFQ/N signaling to the NOP receptor might be critically involved in the

development of morphine tolerance and that other processing products of prepro-OFQ/N may not participate in this process.

Finally, our studies also imply a functional separation of analgesic tolerance and morphine dependence. There are considerable controversies in the literature whether opiate tolerance and physical dependence share a common mechanism. Our experiments show that three weeks of low-dose morphine treatment produced similar level of withdrawal symptoms independent of the level of analgesic tolerance in those animals. On the other hand, mice rendered completely tolerant to the analgesic effects of morphine by using escalating doses displayed different magnitudes of withdrawal signs depending on their OFQ/N genotype. These examples illustrate that analgesic tolerance is not a necessary precondition for opioid dependence and that, on the other hand, complete analgesic tolerance to morphine can be accompanied by different levels of dependence. Recent evidence suggesting a dissociation of tolerance and dependence were obtained from analysis of  $\delta$ -opioid receptor knockout mice and prepro-enkephalin knockout mice (Nitsche et al., 2002). Both types of mice fail to develop analgesic tolerance to morphine while still exhibiting naloxone-precipitated withdrawal behavior after chronic morphine treatment. In contrast, mice lacking the muscarinic acetylcholine M5 receptor showed attenuated morphine dependence but were still able to develop analgesic tolerance (Basile et al., 2002). Furthermore, one report showed that NMDA and AMPA receptor antagonists might be more effective in blocking development of morphine tolerance than dependence (McLemore et al., 1997), although others have shown that NMDA antagonists effectively block both (Trujillo and Akil, 1991). These examples indicate that

analgesic tolerance and morphine dependence might be differentially modulated by various neuronal systems. In view of our present data it will also be important to examine the influence of morphine dosage regimen in these behavioral and pharmacological models.

In summary, we have shown that blockade of OFQ/N signaling can prevent development of morphine tolerance for a prolonged time when low doses of morphine are used. The use of repeated low doses of morphine is similar to clinical settings where physicians aim to administer the lowest possible effective dose of morphine to ensure alleviation of pain. Our results indicate that OFQ/N antagonists could have therapeutic potential as adjuvants in an opioid-based analgesic therapy by preventing development of morphine tolerance with significant benefits for the long-term treatment of chronic or severe pain, for example in cancer patients.

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### Footnotes

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### Figure Legends:

**Fig 1.** Dose-response of acute morphine administration. Nociceptive responses in mutant mice lacking OFQ/N (-/-) and their wildtype (+/+) littermates were measured in the tail-flick test following i.p. administration of increasing doses of morphine. Data are means  $\pm$  SEM. OFQ/N<sup>+/+</sup>, n=12; OFQ/N<sup>-/-</sup>, n=10.

**Fig 2.** OFQ/N knockout mice fail to develop analgesic tolerance after 3 weeks of treatment with repeated daily low doses of morphine. Nociceptive responses in mutant mice lacking OFQ/N (-/-) and their wildtype (+/+) littermates were measured by tail-flick test during chronic morphine treatment. Mice received daily i.p. injections of 10 mg/kg morphine. A control group of OFQ/N<sup>-/-</sup> mice received daily injections of saline. Data are shown as mean latency  $\pm$  SEM. Two-way ANOVA indicated significant main effect of genotype ( $F_{1,240} = 31.62$ ;  $p < 0.0001$ ) and time ( $F_{8,240} = 21.86$ ;  $p < 0.0001$ ) with significant interaction ( $F_{8,240} = 4.15$ ;  $p = 0.00012$ ) in morphine-treated animals. Post-hoc analysis (Bonferroni) revealed significant differences in analgesic responses beginning after 16 days of morphine treatment. \*\*,  $p < 0.01$ , \*\*\*,  $p < 0.001$ . Morphine treatment: OFQ/N<sup>+/+</sup>, n=18; OFQ/N<sup>-/-</sup>, n=14. Saline treatment: OFQ/N<sup>-/-</sup>, n=10.

**Fig 3.** Escalating doses of morphine produce complete analgesic tolerance independent of OFQ/N genotype. Nociceptive responses in mutant mice lacking OFQ/N (-/-) and their wildtype (+/+) littermates during chronic treatment with escalating doses of morphine

were measured by tail-flick. Data are expressed as mean latency  $\pm$  SEM. No significant differences ( $p > 0.05$ , Student's t-test) were detected between the two genotypes.

OFQ/N<sup>+/+</sup>, n=12; OFQ/N<sup>-/-</sup>, n=10.

**Fig 4.** Co-administration of NOP receptor antagonist J-113397 can block analgesic tolerance to morphine. Nociceptive responses in C57BL/6N mice treated daily with J-113397 (20 mg/kg) or saline followed by morphine (10 mg/kg) were measured by tail-flick test 30 min after morphine injection. Control groups of mice received either J-113397 alone or saline injections. Data are means  $\pm$  SEM. Two-way ANOVA indicated significant main effect of drug treatment ( $F_{1,121} = 342.82$ ;  $p < 0.0001$ ) and time ( $F_{8,121} = 146.2$ ;  $p < 0.0001$ ) with significant interaction ( $F_{8,240} = 36.88$ ;  $p < 0.0001$ ) in J-113397/morphine-treated vs. saline/morphine-treated animals. Post-hoc analysis (Bonferroni) revealed significant differences in analgesic responses of J-113397/morphine-treated mice beginning after 11 days of treatment. \*\*\*,  $p < 0.001$ . Saline/morphine-treated group, n=7; J-113397/morphine-treated group, n=9; J-113397/saline-treated group, n=7; saline/saline-treated group, n=8.

**Fig 5.** Naloxone-precipitated withdrawal. *A*, Withdrawal jumps in C57BL/6 mice treated with chronic morphine (10 mg/kg) together with saline or J-113397 for 22 days. Saline-treated group, n=7; J-113397 treated group, n=9. *B*, Withdrawal jumps observed in wildtype (+/+) and OFQ/N knockout mice (-/-) after chronic morphine administration (10 mg/kg) for 22 days. OFQ/N<sup>+/+</sup>, n=10; OFQ/N<sup>-/-</sup>, n=8. *C*, Withdrawal jumps in wildtype

(+/+) and OFQ/N knockout (-/-) mice treated with escalating doses of morphine.

OFQ/N<sup>+/+</sup>, n=14; OFQ/N<sup>-/-</sup>, n=17. All data are presented as means  $\pm$  S.E.M.

Fig. 1

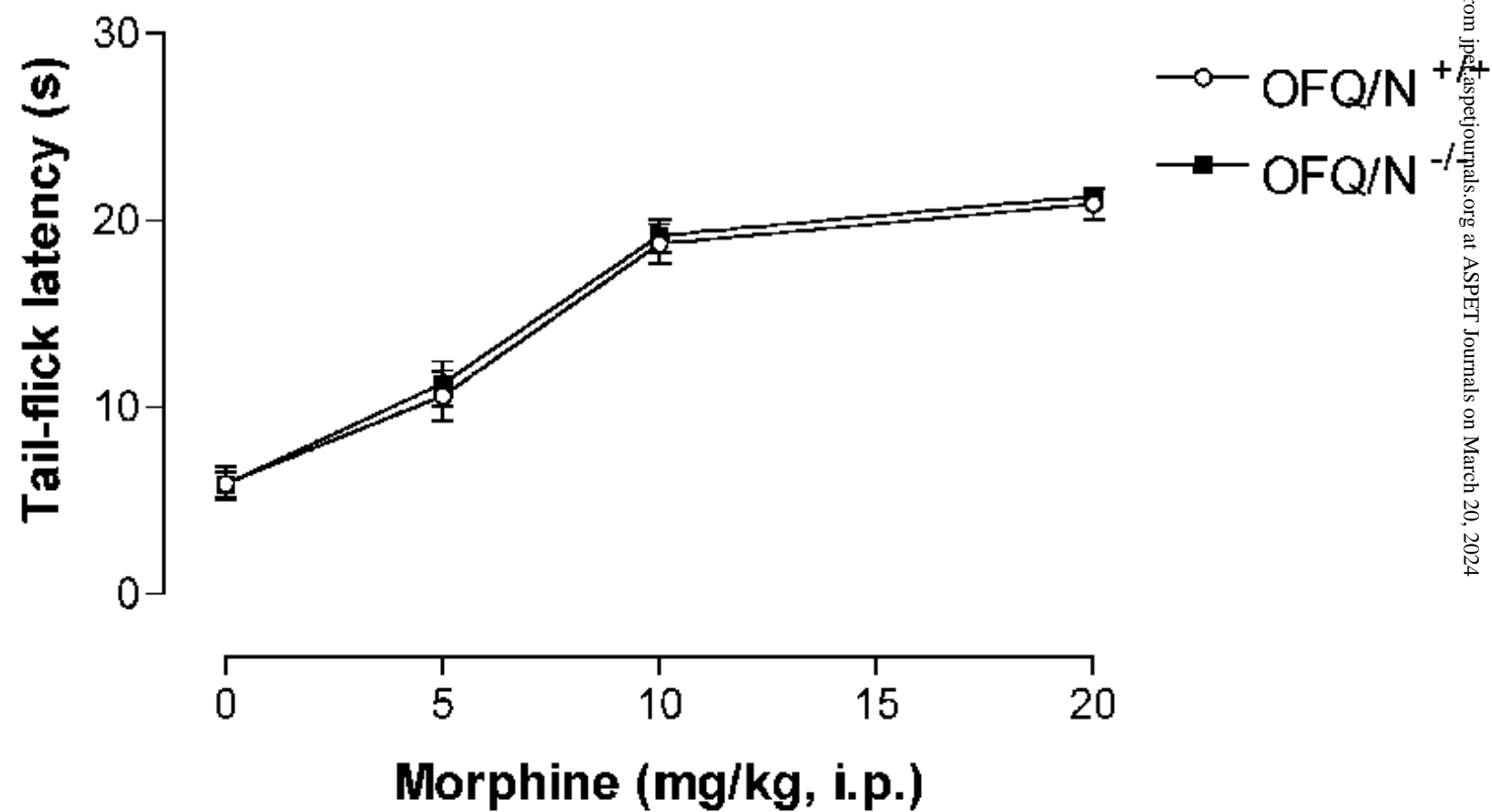


Fig. 2

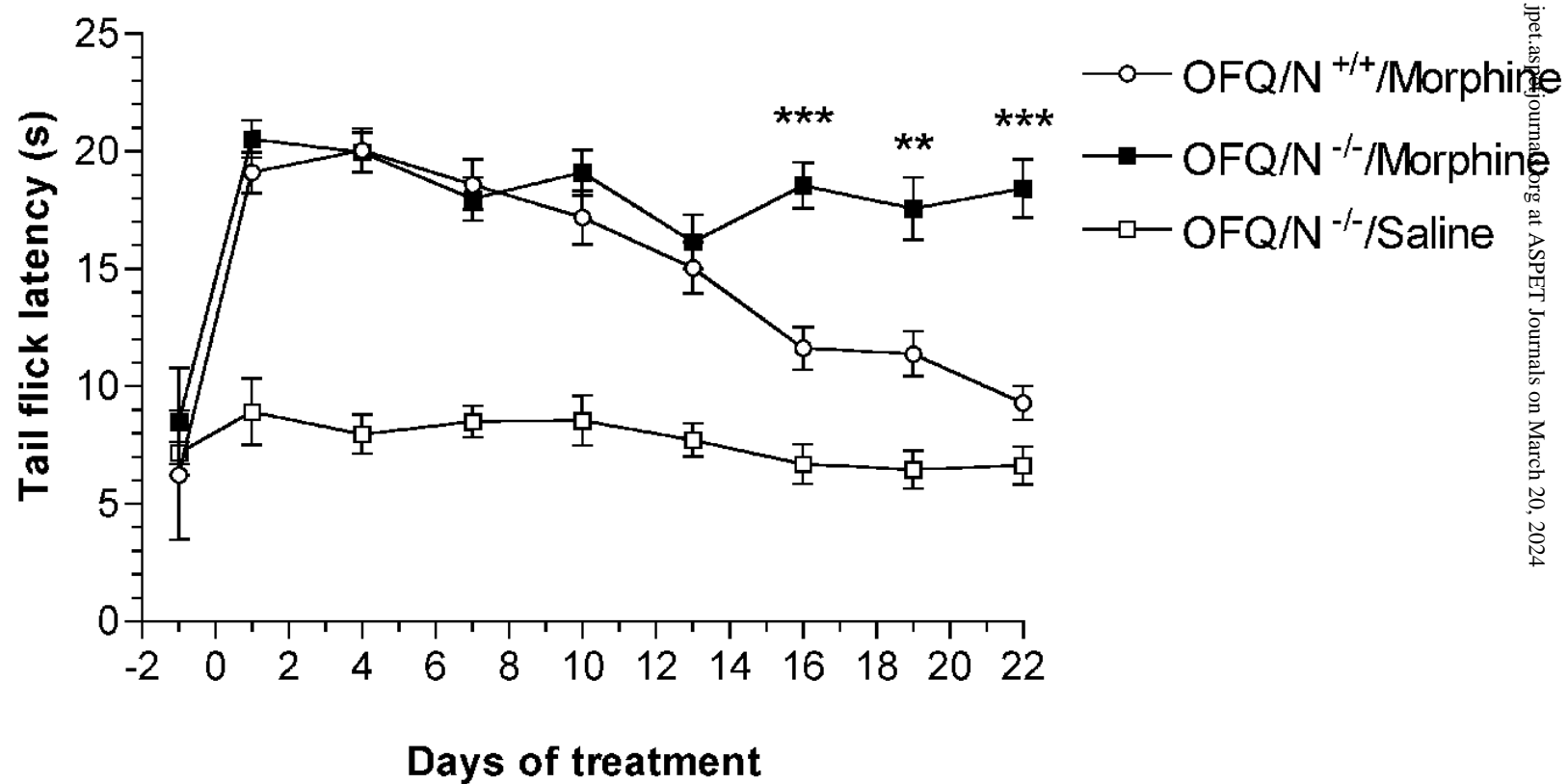




Fig. 3

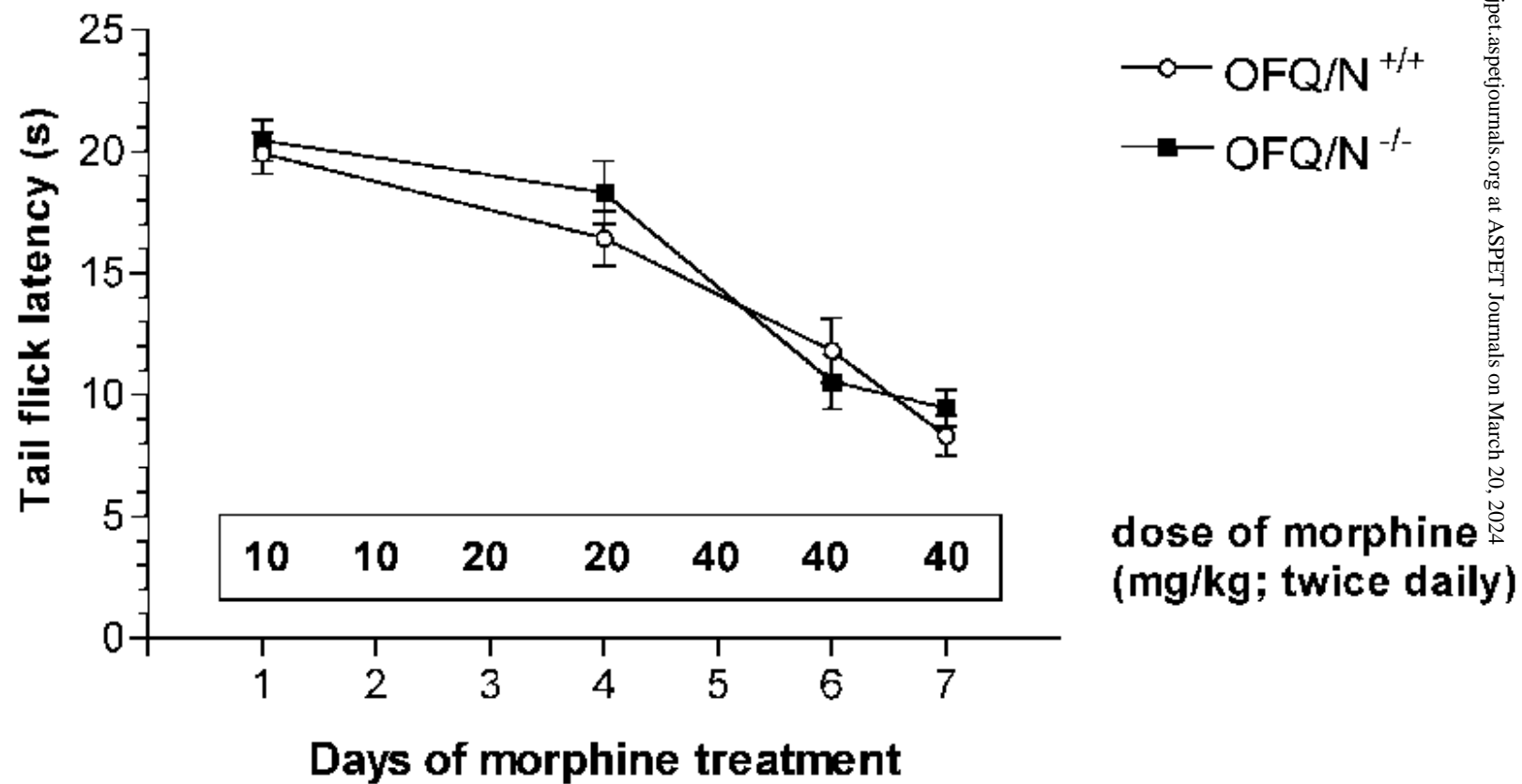


Fig. 4

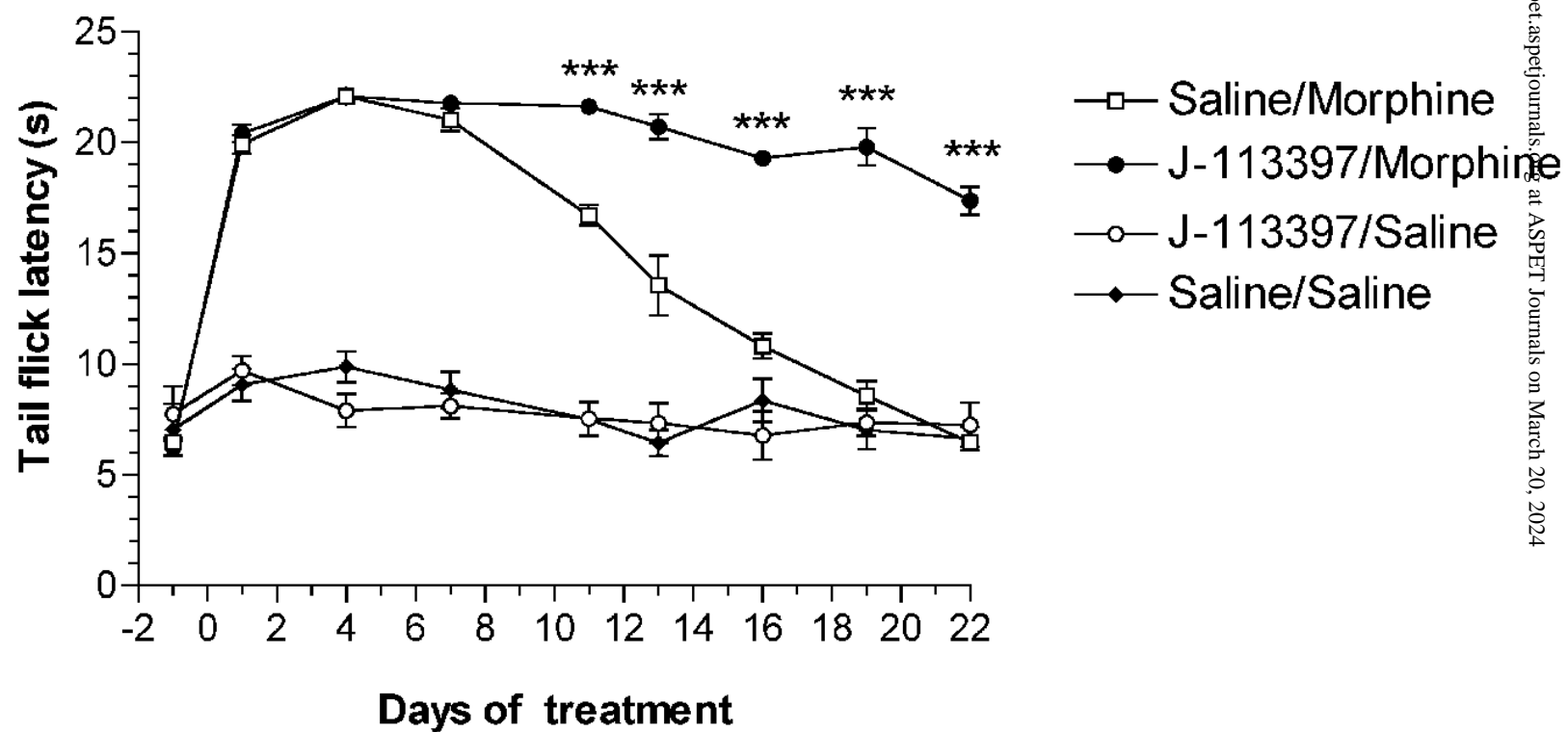


Fig. 5

