



Full length article

Involvement of the N/OFQ-NOP system in rat morphine antinociceptive tolerance: Are astrocytes the crossroad?



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ABSTRACT

The development of tolerance to the antinociceptive effect is a main problem associated with the repeated administration of opioids. The progressively higher doses required to relieve pain reduce safety and exacerbate the side effects of classical opioid receptor agonists like morphine. Nociceptin/orphanin FQ (N/OFQ) and its NOP receptor constitute the fourth endogenous opioid system that is involved in the control of broad spectrum of biological functions, including pain transmission. Aim of this work was to evaluate the relevance of the N/OFQ-NOP system in morphine antinociceptive action and in the development of morphine tolerance in the rat. Continuous spinal intrathecal infusion of morphine (1–3 nmol/h) evoked analgesic effects for 5 days in wild type animals. The same doses infused in NOP(-/-) rats showed a lower analgesic efficacy, while the onset of tolerance was delayed to day 9. N/OFQ (1–3 nmol/h), continuously infused in NOP(+/+) animals, showed an analgesic profile similar to morphine. Immunohistochemical analysis of the dorsal horn of the spinal cord of morphine tolerant NOP(+/+) rats showed an increased number of Iba1- and GFAP-positive cells (microglia and astrocytes, respectively). Interestingly, microglia but not astrocyte activation was observed in NOP(-/-) morphine tolerant rat. A selective activation of astrocytes was observed in the dorsal horn of wild type N/OFQ tolerant rats.

The antinociceptive effect of morphine partially depends by the N/OFQ-NOP system that participates in the development of morphine tolerance. In particular, NOP receptors are involved in morphine-induced astrocyte activation, and N/OFQ *per se* increases astrocyte density.

1. Introduction

Morphine is one of the most powerful drug used in pain relief and remains a preferred and commonly prescribed analgesic for pain management (Denlinger et al., 2014). Although it shows remarkable efficacy for treating chronic pain conditions, there are several limitations to its use (Yuill et al., 2016), including rapid development of tolerance, which is characterized by a decrease in antinociceptive efficacy (Self and Nestler, 1995). To overcome tolerance and achieve equivalent pain relief, the opioid dose is progressively escalated which can exacerbate the unwanted effects (Thorn et al., 2016). Mechanisms of tolerance are complex and involves changes in receptors, ion channels cell and neuronal networks functions (Ji, 2010; Johansson et al., 2010; Zhang et al., 2015). According to a classical view these rearrangements occur at the neuronal level, however growing evidence indicates a potential role of glia cells that are activated by repeated administrations of morphine (Di Cesare Mannelli et al., 2015; Horvath

and DeLeo, 2009; Mika, 2008; Mika et al., 2009; Raghavendra et al., 2002; Song and Zhao, 2001; Watkins et al., 2005). Morphine treatment induces astrocyte and microglia changes resulting in increased production of many soluble factors such as proinflammatory cytokines and chemokines, neurotrophic factors, prostaglandins, nitric oxide, complement proteins, free radicals, neurotoxins and excitatory amino acids that counteract opioid analgesia (Eidson and Murphy, 2013; Hameed et al., 2010). The pharmacological inhibition of microglia and astrocyte with minocycline and fluorocitrate, respectively, attenuates the onset of tolerance in rats (Cui et al., 2008; Song and Zhao, 2001).

Nociceptin/orphanin FQ (N/OFQ), a heptadecapeptide from the opioid family, and its cognate NOP receptor (previously called the opioid receptor-like, ORL1), are present in nociceptive pathways in spinal cord and brain with a complex role (Schröder et al., 2014; Zeilhofer and Calò, 2003). N/OFQ differentially modulates nociception depending upon site of administration, dose, assay, and animal species. N/OFQ injected intracerebroventricularly (i.c.v) decreases the pain

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threshold in the hot-plate and tail-flick assays (Meunier et al., 1995; Reinscheid et al., 1995). Moreover, after the i.c.v. administration, N/OFQ attenuates morphine analgesia (Mogil et al., 1996). On the contrary, intrathecal (i.t.) administration of the peptide shows acute antinociceptive activity in the tail-flick and in the paw pressure tests (Micheli et al., 2015a; Xu et al., 1996). Similarly, in models of neuropathic and inflammatory pain, i.t. injections of N/OFQ have also been shown to produce anti-hyperalgesic (Micheli et al., 2015b) and anti-allodynic effects and to potentiate morphine anti-hyperalgesia (Courteix et al., 2004; Hao et al., 1998; Yamamoto et al., 1997). Despite these studies prove the properties of N/OFQ to evoke antinociceptive effect after spinal administration, no definitive data are reported regarding the involvement of the NOP system in the onset of opioid analgesic morphine tolerance.

Aiming to evaluate the involvement of the NOP system in the onset of morphine tolerance, morphine was administered in NOP(+/+) and NOP(-/-) animals through an intrathecal catheter that allowed a continuous infusion of the compounds at the lumbar level of the spinal cord. The glial activation profile was studied in the dorsal horn in comparison to the same treatment with N/OFQ.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley and Wistar rats were supplied by Envigo (Varese, Italy). Male NOP(-/-) rats, supplied by GenOway (Lyon, France), were generated in a Brown Norway background and subsequently backcrossed on a Wistar background for four generations as previously described in detail (Homberg et al., 2009). For knockout studies Wistar rats were used as controls and indicated as NOP(+/+). Animals, weighing approximately 280–300 g at the beginning of the experimental procedure, were housed in Ce.S.A.L (Centro Stabilazione Animali da Laboratorio, University of Florence) and used at least one week after their arrival. Four rats were housed per cage (size 26 × 41 cm) kept at 23 ± 1 °C with a 12 h light/dark cycle, light at 7 a.m.; animals were fed with standard laboratory diet and tap water *ad libitum*. All animal manipulations were carried out according to the Directive 2010/63/EU of the European parliament and of the European Union council (22 September 2010) on the protection of animals used for scientific purposes. The ethical policy of the University of Florence complies with the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health (NIH Publication No. 85-23, revised 1996; University of Florence assurance number: A5278-01). Formal approval to conduct the experiments described was obtained from the Animal Subjects Review Board of the University of Florence. Experiments involving animals have been reported according to ARRIVE guidelines (McGrath and Lilley, 2015). All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2. Intrathecal catheterization

Rats were anesthetized with 2% isoflurane and intrathecal catheter was surgically implanted according to the Yaksh and Rudy (1976) method. Rats were shaved on the back of the neck and placed in the stereotaxic frame with the head securely held between ear bars. The skin over the nap of the neck was cleaned with ethyl alcohol and incised for 1 cm. The muscle on either side of the external occipital crest was detached and retracted to expose about 3–4 mm² of the atlanto-occipital membrane. The membrane was incised by a needle, which led to the escape of cerebrospinal fluid. The caudal edge of the cut was lifted and about 7.0 cm of 28G polyurethane catheter (0.36 mm outer diameter; 0.18 mm inner diameter; Alzet, USA) was gently inserted into the intrathecal space in the midline, dorsal to the spinal cord until the lumbar enlargement. The exit end of the catheter was connected to the osmotic pumps (Alzet, USA) for continuous infusion. Then pumps were

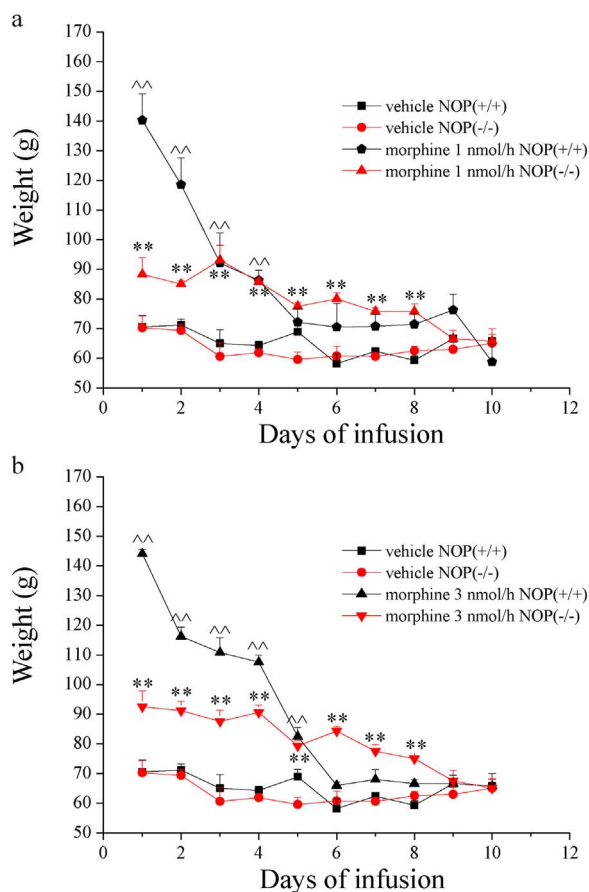


Fig. 1. Analgesic effect induced by continuous intrathecal infusion of morphine in NOP(+/+) and in NOP(-/-) rats. a) The opioid compound was dissolved in sterile saline solution and infused in two different groups of animals both at the dose of 1 nmol/h with osmotic pumps until day 10. Vehicle NOP(+/+) and vehicle NOP(-/-) groups were infused with sterile saline solution. The nociceptive threshold was evaluated every day by Paw pressure test, starting on the 1st day of treatment. b) The opioid compound was dissolved in sterile saline solution and infused in two different groups of animals, both at the dose of 3 nmol/h with osmotic pumps until day 10. Vehicle NOP(+/+) and vehicle NOP(-/-) groups were infused with sterile saline solution. The nociceptive threshold was evaluated every day by Paw pressure test, starting on the 1st day of treatment. Each value represents the mean of 8 rats performed in 2 different experimental sets. Data are shown as mean ± S.E.M; ~P < 0.01 vs vehicle NOP(+/+) infused rats; **P < 0.01 vs vehicle NOP(-/-) infused rats.

subcutaneously fixed on the back of the head. The incision site in the skin was sutured with polyamide wire and animals were allowed to recover for 24 h before the study began.

All animals used during behavioral tests did not show motor impairment induced by surgical operation for the catheter implantation. The evaluation of potential motor dysfunctions were investigated using the Rota rod test (Ugo Basile, Varese, Italy). The animals that presented any kind of motor disability (approximately 15%) were excluded from the behavioral measurements.

2.3. Drug intrathecal treatments

Morphine hydrochloride was from S.A.L.A.R.S., Como, Italy. N/OFQ was synthesized and purified as previously described in details (Arduin et al., 2007; Guerrini et al., 1997). All compounds were dissolved in sterile saline solution.

For continuous infusion, compound concentrations were adjusted to release 1 and 3 nmol/h morphine or N/OFQ from a mini-osmotic pump ALZET 1002 (USA) with a flow rate 0.25 µL/h. Final solutions were filtered by a 0.22 µm pore size Hydrophilic PVDF membrane (Millipore, Italy). Mini-pumps were attached to the described polyurethane

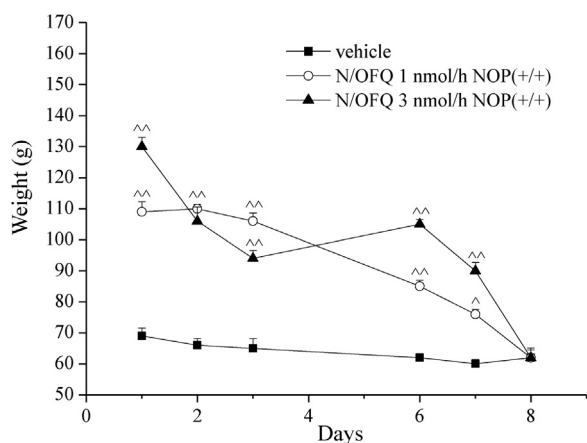


Fig. 2. Effect induced by continuous intrathecal infusion of N/OFQ in NOP (+/+) rats. The compound was dissolved in sterile saline solution and administered in two different groups of animals at the dosages of 1 and 3 nmol/h with osmotic pumps until day 8. Vehicle NOP (+/+) group was infused with sterile saline solution. The nociceptive threshold was evaluated by Paw pressure test, starting the 1st day of treatment. Each value represents the mean of 8 rats performed in 2 different experimental sets. Data are shown as mean \pm S.E.M.; * $P < 0.05$ and ** $P < 0.01$ vs vehicle NOP (+/+) infused rats.

catheters and filled with compounds or vehicle solutions, respectively. All mini-osmotic pumps were incubated in sterile saline solution overnight at 37 °C.

2.4. Paw pressure test

The nociceptive threshold in the rat was determined with an analgesiometer (Ugo Basile, Varese, Italy), according to the method described by Leighton et al. (1988). Briefly, a constantly increasing pressure was applied to a small area of the dorsal surface of the paw using a blunt conical probe by a mechanical device. Mechanical pressure was increased until vocalization or a withdrawal reflex occurred while rats were lightly restrained. Vocalization or withdrawal reflex thresholds were expressed in grams. Rats scoring below 35 g or over 75 g during the test before the surgical procedure for the catheter implantations were rejected. For analgesia measures, mechanical pressure application was stopped at 180 g. All experiments were performed by a researcher blind to drug treatments.

2.5. Rota-rod test

The Rota-rod apparatus (Ugo Basile, Varese, Italy) consisted of a base platform and a rotating rod with a diameter of 6 cm and a non-slippery surface. The rod was placed at a height of 25 cm from the base. The rod, 36 cm in length, was divided into 4 equal sections by 5 disks. Thus, up to 4 rats were tested simultaneously on the apparatus, with a rod-rotating speed of 10 r.p.m. The integrity of motor coordination was assessed on the basis of the time the animals kept their balance on the rotating rod up to a maximum of 120 s. The number of falls from the rod was also measured. After a maximum of 6 falls, the test was suspended and the time was recorded.

2.6. Immunofluorescence staining

After the disappearance of the antinociceptive effects (day 6 for morphine-treated NOP(+/+); day 9 for morphine-treated NOP(-/-); day 8 for morphine-treated NOP(+/+)) animals from each treatment group were killed using a guillotine. The L4/L5 segments of the spinal cord were exposed from the lumbar vertebral column via laminectomy and identified by tracing the dorsal roots from their respective dorsal root ganglia (DRG). As previously described by (Di Cesare Mannelli et al., 2013, 2014; Tomassoni et al., 2013), formalin-fixed cryostat

sections (7 μ m) were washed 3 \times phosphate-buffered saline (PBS), 0.3% Triton X-100 for 5 min and then were incubated, at room temperature, for 1 h in blocking solution (PBS, 0.3% Triton X-100, 5% albumin bovine serum; PBST). Slices were incubated overnight at 4 °C in PBST containing rabbit primary antisera. The primary antibody used was directed against ionized calcium binding adapter molecule 1 (Iba1; rabbit, 1:1000; Wako, Richmond, VA, USA) for microglial staining or against glial fibrillary acidic protein (GFAP; rabbit, 1:1000; DAKO, Carpinteria, CA, USA) for astrocyte staining. The following day, slides were washed 3 \times PBS, 0.3% Triton X-100 for 5 min and then sections were incubated in goat anti-rabbit IgG secondary antibody labeled with Alexa Fluor 568 (1:500; Invitrogen, Carlsbad, USA) and DAPI (4', 6-diamidin-2-fenilindolo; 1:2000; Life Technologies-Thermo scientific, Rockford, IL, USA), a nuclei marker, in PBST at room temperature for 2 h, in the dark. After 3 \times PBS 0.3% Triton X-100 wash for 10 min, slices were mounted using ProLong Gold (Life Technologies-Thermo scientific, Rockford, IL, USA) as a mounting medium.

Negative control sections (no exposure to the primary antisera) were processed concurrently with the other sections for all immunohistochemical studies, in order to exclude the presence of non-specific immunofluorescent staining or cross-immunostaining. Images were acquired by using an Olympus BX63 microscope equipped with an Olympus XM10 camera and coupled to CellSense Dimension Software (Olympus, Milan, Italy).

Quantitative analysis of GFAP and Iba1-positive cells was performed by collecting three independent fields through a 20 \times 0.40NA objective in the dorsal horn of each rat spinal cord. GFAP and Iba1-positive cells were counted using the “cell counter” plugin of ImageJ (NIH, Bethesda, Maryland, USA).

2.7. Statistical analysis

Behavioral measurements were performed on 8 rats for each treatment carried out in 2 different experimental sets. Results were expressed as means \pm S.E.M. and the analysis of variance was performed by two way ANOVA. A Bonferroni's significant difference procedure was used as post-hoc comparison. P values of less than 0.05 or 0.01 were considered significant. Data were analyzed using the “Origin 9” software (OriginLab, Northampton, USA).

3. Results

We compared the analgesic effects of morphine (1 nmol/h and 3 nmol/h) induced by a continuous intrathecal (i.t.) infusion in NOP (+/+) and NOP(-/-) rats. Control groups were treated with sterile saline solution. To allow a constant delivery of the substance over days, mini-osmotic pumps connected to catheters were used. Pain threshold was evaluated every day by Paw pressure test, beginning 24 h after pump implant (Fig. 1A and B). Animals treated with vehicle displayed a stable pain threshold over the time course of the experiment (10 days). No difference in the basal pain threshold between NOP (+/+) and NOP (-/-) rats was highlighted.

On day 1, morphine elicited a higher antinociceptive effect in NOP (+/+) animals in comparison to the same dose infused in NOP(-/-) rats. A superior effect in NOP(+/+) rats was maintained for 2 and 4 days depending on the treatment with 1 or 3 nmol/h, respectively. In NOP(+/+) rats morphine effectiveness rapidly decreased (dose-dependently) losing significance on day 5 (Fig. 1A and B). In NOP(-/-) animals, the antinociceptive effect of morphine, even lower, was more stable and longer lasting for both dosages used (1 and 3 nmol/h). Efficacy was lost on day 9 (Fig. 1A and B).

Results obtained by the continuous intrathecal infusion of the endogenous peptide N/OFQ (1 and 3 nmol/h) in NOP(+/+) rats are shown in Fig. 2. N/OFQ efficacy was slightly lower than morphine on day 1 but the decrease in the following days was lesser drastic. Moreover, the antinociceptive effect was maintained till day 7. As previously

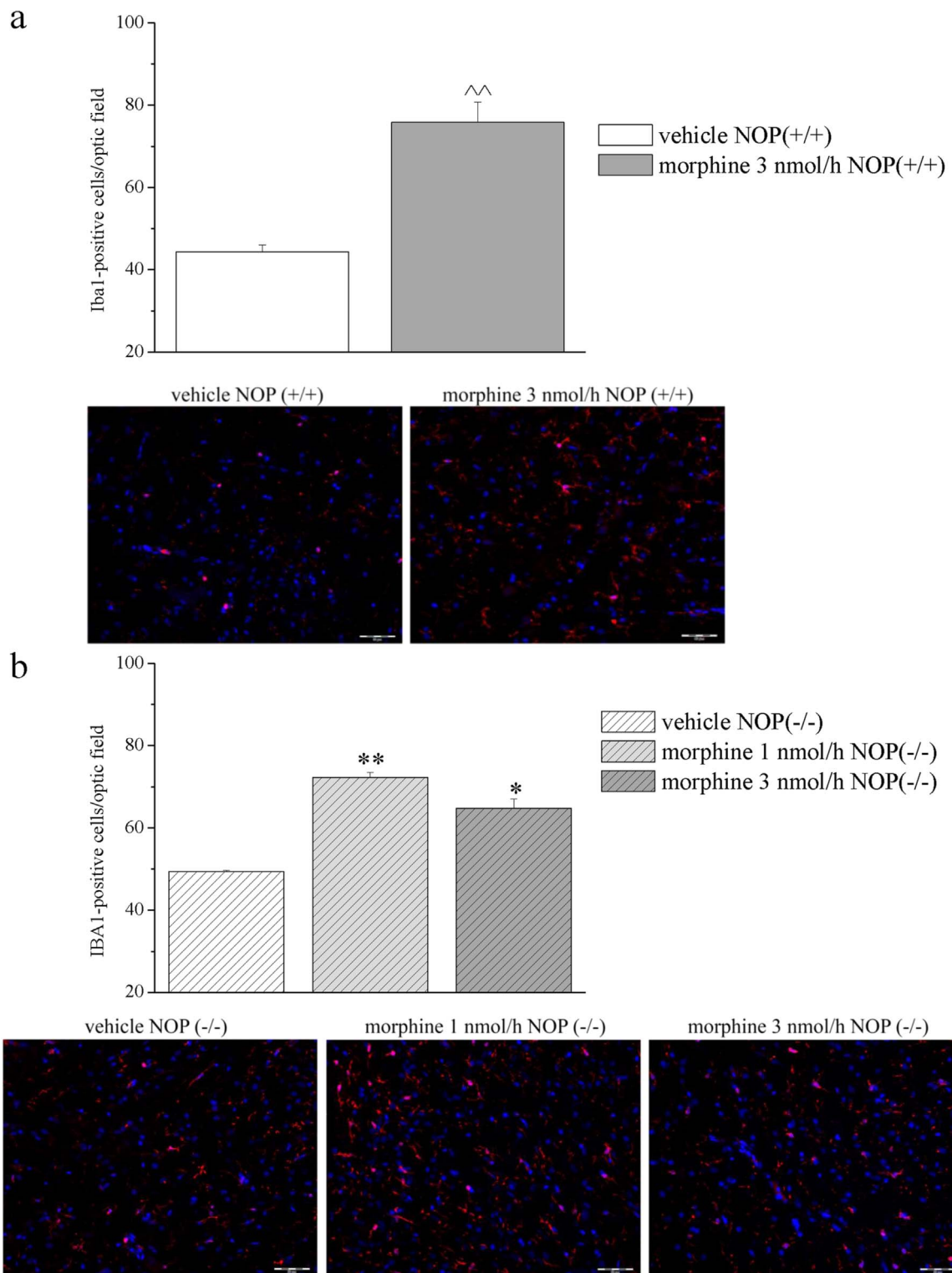


Fig. 3. Iba1-positive cell density in the dorsal horn of the spinal cord of morphine NOP(+/+) and NOP(-/-) tolerant rats. **a)** Morphine 3 nmol/h was continuously intrathecal infused in NOP(+/+) rats until the onset of tolerance and immunohistochemical analysis was performed on day 6. Representative images of merged Iba1-labeled microglia cells (red), plus DAPI-labeled cell nuclei (blue), scale bar: 50 μ m. **b)** Morphine 1 nmol/h and 3 nmol/h was continuously intrathecal infused in NOP(-/-) rats until the onset of tolerance and immunohistochemical analysis was performed on day 9. Representative images of merged Iba1-labeled microglia cells (red), plus DAPI-labeled cell nuclei (blue), scale bar: 50 μ m. Each value represents the mean of 4 rats performed in 2 different experimental sets. Data are shown as mean \pm S.E.M.; \sim P < 0.01 vs vehicle NOP(+/+) infused rats. *P < 0.05 and **P < 0.01 vs vehicle NOP(-/-) infused rats. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

reported, N/OFQ does not induce analgesia in NOP(-/-) animals (Micheli et al., 2015a).

After the disappearance of the antinociceptive effects (day 6 for morphine-treated NOP(+/+) at the dose of 3 nmol/h; day 9 for

morphine-treated NOP(-/-) at both dosages (1 and 3 nmol/h); day 8 for nociceptin/OFQ-treated NOP(+/+) animals from each treatment group were killed for spinal cord collection. The dorsal horn were collected and glial cells analyzed by immunohistochemistry at the

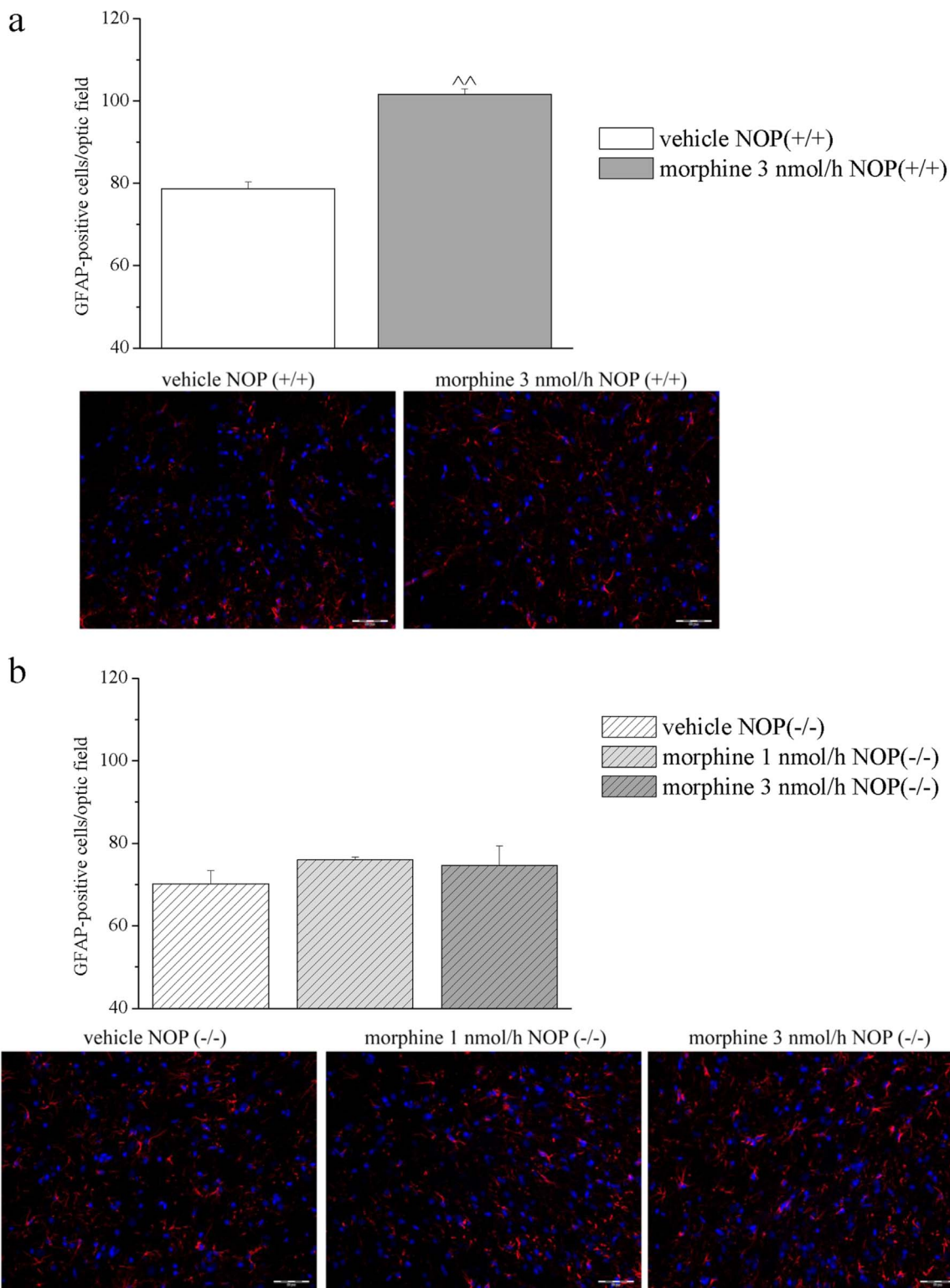


Fig. 4. GFAP-positive cell density in the dorsal horn of the spinal cord of morphine NOP(+/+) and NOP(-/-) tolerant rats. a) Morphine 3 nmol/h was continuously intrathecal infused in NOP(+/+) rats until the onset of tolerance and immunohistochemical analysis was performed on day 6. Representative images of merged GFAP-labeled astrocytes cells (red), plus DAPI-labeled cell nuclei (blue), scale bar: 50 μ m. b) Morphine 1 nmol/h and 3 nmol/h was continuously intrathecal infused in NOP(-/-) rats until the onset of tolerance and immunohistochemical analysis was performed on day 9. Representative images of merged GFAP-labeled astrocytes cells (red), plus DAPI-labeled cell nuclei (blue), scale bar: 50 μ m. Each value represents the mean of 4 rats performed in 2 different experimental sets. Data are shown as mean \pm S.E.M.; *P < 0.01 vs vehicle NOP(+/+) infused rats. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

lumbar level. In wild type animals, morphine, infused at the dose of 3 nmol/h, increased the number of both Iba1- and GFAP-positive cells in comparison to control (Figs. 3A and 4A). Differently, the microglial component only was increased by morphine (1 and 3 nmol/h) in NOP

(-/-) rats whereas astrocytes were unmodified by treatment (Figs. 3B and 4B).

In Fig. 5, the effect of N/OFQ on glial cells is shown. The intrathecal infusion of N/OFQ (1 nmol/h and 3 nmol/h) did not alter the number of

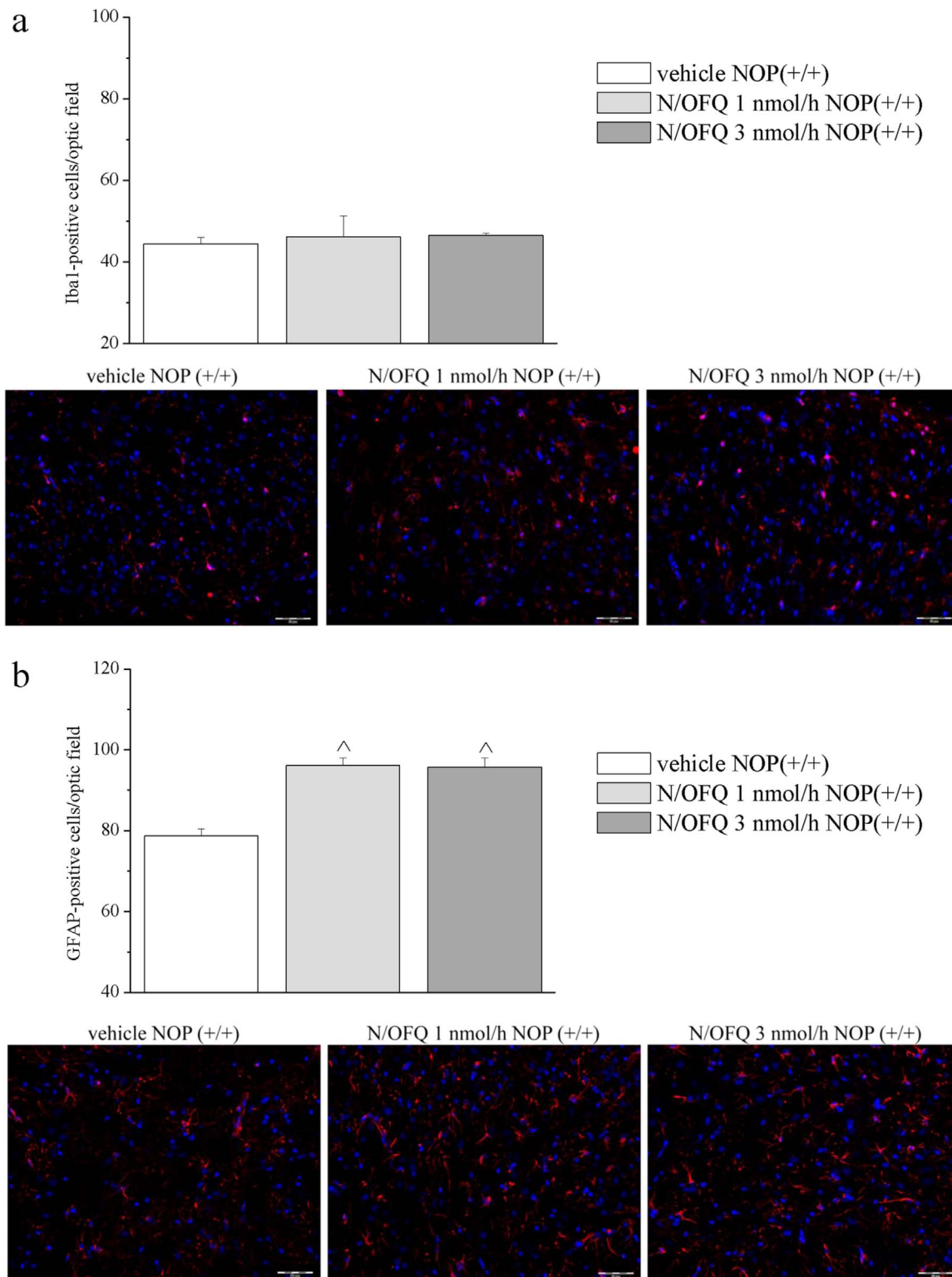


Fig. 5. Iba1-positive cell density and GFAP-positive cell density in the dorsal horn of the spinal cord of N/OFQ NOP(+/+) tolerant rats. a) N/OFQ 1 nmol/h and 3 nmol/h was continuously intrathecal infused in NOP(+/+) rats until the onset of tolerance and immunohistochemical analysis were performed on day 8. Representative images of merged Iba1-labeled microglia cells (red), plus DAPI-labeled cell nuclei (blue), scale bar: 50 μ m. b) N/OFQ 1 nmol/h and 3 nmol/h was continuously intrathecal infused in NOP(+/+) rats until the onset of tolerance and immunohistochemical analysis were performed on day 4. Representative images of merged GFAP-labeled astrocytes cells (red), plus DAPI-labeled cell nuclei (blue), scale bar: 50 μ m. Each value represents the mean of 8 rats performed in 2 different experimental sets. Data are shown as mean \pm S.E.M; [^]P < 0.05 vs vehicle NOP(+/+) infused rats. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

Iba-1 positive cells (Fig. 5B). On the contrary, the density of GFAP-positive cells was significantly increased (Fig. 5B).

4. Discussion

Results showed that genetic deletion of NOP receptor is associated with reduction in the analgesic effect and tolerance development of morphine. Knocking out the NOP receptor, morphine is less efficient in increasing the pain threshold but the effect is longer lasting in comparison with wild type animals. Moreover, immunohistochemistry revealed that lack of NOP receptor is also associated with reduced activation of GFAP-positive cells occurring in morphine tolerant rats indicating a potential role of NOP/OFQ system in regulation of tolerance through modulation of astrocytes-related mechanisms. This hypothesis is further supported by results in wild type rats in which N/OFQ infusion was associated with activation of astrocytes.

The lower analgesic efficacy of morphine in NOP(-/-) rats agree with our previous evidence after an acute intrathecal injections in rats (Micheli et al., 2015a). Conversely, other authors reported similar analgesic effects of morphine in NOP(+ / +) and NOP(- / -) in mice (Mamiya et al., 2001; Ueda et al., 1997) suggesting a different role of N/OFQergic signaling in rats and mice. On the other hand, a partial loss of tolerance liability to morphine analgesia was observed also in mice lacking the NOP receptor gene (Ueda et al., 1997).

The development of morphine tolerance and dependence is also associated with changes of the NOP receptor system. For instance, in wild type mice, chronic administration of morphine induced an increase of NOP gene expression in the spinal cord while the administration of J-113397, a non-peptide N/OFQ antagonist (Bigoni et al., 2000; Ozaki et al., 2000), prevented the development of morphine tolerance (Caputi et al., 2014; Chung et al., 2006; Ueda et al., 2000). Accordingly, the concentration of endogenous N/OFQ increased in the rat ventricular cerebrospinal fluid, in periaqueductal gray and amygdala in a time-dependent manner during the development of morphine tolerance (Yuan et al., 1999).

During the development of morphine tolerance similar increases were observed also for cholecystokinin (Becker et al., 2000; Lucas et al., 1999) and neuropeptide FF (Malin et al., 1990), both of which have been shown to contrast the analgesic effects of opioid in the central nervous system. Hence, engagement of antiopioid peptide systems may represent a key mechanism responsible for the development of tolerance to the analgesic effects of morphine (Harrison et al., 1998).

The demonstration that N/OFQ blocks opioid receptor-mediated stress-induced antinociception (Mogil et al., 1996) and also decreases the antinociceptive effect of morphine and other opioid receptor agonists (Lutfy et al., 2001; Mogil et al., 1996; Morgan et al., 1997; Tian et al., 1997) has led to the suggestion that N/OFQ may act as an antiopioid peptide in the brain (Mogil et al., 1996).

Among several mechanisms proposed to explain morphine tolerance, the activation of glial cells assumes a main role (Cui et al., 2008; Eidson and Murphy, 2013; Ferrini et al., 2013; Song and Zhao, 2001). To the best of our knowledge, there are no findings regarding the relationship between glial cells and N/OFQ in the onset of morphine tolerance.

In the present study we reported the glial response in the dorsal horn of the spinal cord in NOP(+ / +) and NOP(- / -) rats tolerant to morphine. We performed the same analysis in NOP(+ / +) rats tolerant to N/OFQ. Wild type animals, tolerant to morphine after the intrathecal continuous infusion of the alkaloid at the dose of 3 nmol/h, showed glial activation characterized by an increase in cell density without morphological alterations of both microglia and astrocytes. This results are in accord with previous evidence (Di Cesare Mannelli et al., 2015). On the contrary, in wild type rats tolerant to N/OFQ the number of astrocyte cells is statistically increased whereas microglia density is not altered. Nevertheless, when the NOP receptor is deleted morphine treatment selectively activates microglia but not astrocytes. A NOP-

mediated morphine-induced astrocyte activation may be hypothesized. On the other hand, fluorocitrate, a nonselective metabolic inhibitor of astrocytes, partially reversed the development of morphine tolerance in rats (Song and Zhao, 2001). Moreover, astrocytes express NOP receptor and cytokine production by astrocytes can be inhibited by N/OFQ, an effect which represents one mechanism by which N/OFQ may reduce pain sensitivity (Fu et al., 2007). Furthermore, N/OFQ mRNA is increased in astrocytes treated with morphine (Takayama and Ueda, 2005), further, various proinflammatory cytokines as those increased during morphine treatment (eg., tumor necrosis factor and interleukin-1) can enhance astrocyte concentrations of immunoreactive N/OFQ and N/OFQ mRNA (Buzas et al., 2002; Mallimo and Kusnecov, 2013). In this view, our data suggest that lacking NOP receptor the ancillary role of N/OFQ in morphine antinociception is lost but losing the NOP-dependent astrocyte signal tolerance development may be delayed. The topic deserves further studies to clarify the role of N/OFQ signaling in astrocyte and to attempt a NOP modulation for enhancing morphine antinociceptive activity. In particular, an in vivo study with the continuous i.t. infusion of morphine and a NOP receptor antagonist will be performed to highlight the link between the onset of morphine tolerance and the NOP system.

5. Conclusion

In conclusion the antinociceptive effect of morphine seems to be partially dependent from the NOP system since rats lacking NOP receptor are less sensitive to the analgesic effect of this opioid agonist. Interestingly, these results revealed also that receptor deletion contributes to delay the development of morphine tolerance possibly through mechanisms associated with astrocytes activation.

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Conflict of interest statement

All authors declare no conflict of interest.

References

- Arduin, M., Spagnolo, B., Calò, G., Guerrini, R., Carrà, G., Fischetti, C., Trapella, C., Marzola, E., McDonald, J., Lambert, D.G., Regoli, D., Salvadori, S., 2007. Synthesis and biological activity of nociceptin/orphanin FQ analogues substituted in position 7 or 11 with Calpha, alpha-dialkylated amino acids. *Bioorg. Med. Chem.* 15 (13), 4434–4443.
- Becker, C., Pohl, M., Thiébot, M.H., Collin, E., Hamon, M., Cesselin, F., Benoliel, J.J., 2000. Delta-opioid receptor-mediated increase in cortical extracellular levels of cholecystokinin-like material by subchronic morphine in rats. *Neuropharmacology* 39 (2), 161–171.
- Bigoni, R., Calò, G., Rizzi, A., Guerrini, R., De Risi, C., Hashimoto, Y., Hashiba, E., Lambert, D.G., Regoli, D., 2000. In vitro characterization of J-113397, a non-peptide nociceptin/orphanin FQ receptor antagonist. *Naunyn. Schmiede. Arch. Pharmacol.* 361 (5), 565–568.
- Buzas, B., Rosenberger, J., Kim, K.W., Cox, B.M., 2002. Inflammatory mediators increase the expression of nociceptin/orphanin FQ in rat astrocytes in culture. *Glia* 39, 237–246.
- Caputi, F.F., Di Benedetto, M., Carretta, D., Bastias del Carmen, Candia, S., D'Addario, C., Cavina, C., Candeletti, S., Romualdi, P., 2014. Dynorphin/KOP and nociceptin/NOP gene expression and epigenetic changes by cocaine in rat striatum and nucleus accumbens. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 49, 36–46.
- Chung, S., Pohl, S., Zeng, J., Civelli, O., Reinscheid, R.K., 2006. Endogenous orphanin FQ/nociceptin is involved in the development of morphine tolerance. *J. Pharmacol. Exp. Ther.* 318, 262–267.
- Courteix, C., Coudoré-Civiale, M.A., Privat, A.M., Péliissier, T., Eschalier, A., Fialip, J., 2004. Evidence for an exclusive antinociceptive effect of nociceptin/orphanin FQ, an endogenous ligand for the ORL1 receptor, in two animal models of neuropathic pain. *Pain* 110 (1–2), 236–245.
- Cui, Y., Liao, X.X., Liu, W., Guo, R.X., Wu, Z.Z., Zhao, C.M., Chen, P.X., Feng, J.Q., 2008.

- A novel role of minocycline: attenuating morphine antinociceptive tolerance by inhibition of p38 MAPK in the activated spinal microglia. *Brain Behav. Immun.* 22 (1), 114–123.
- Denlinger, C.S., Ligibel, J.A., Are, M., Baker, K.S., Demark-Wahnefried, W., Friedman, D.L., Goldman, M., Jones, L., King, A., Ku, G.H., Kvale, E., Langbaum, T.S., Leonard-Warren, K., McCabe, M.S., Melisko, M., Montoya, J.G., Mooney, K., Morgan, M.A., Moslehi, J.J., O'Connor, T., Overholser, L., Paskett, E.D., Raza, M., Syrjala, K.L., Urba, S.G., Wakabayashi, M.T., Zee, P., McMillian, N., Freedman-Cass, D., 2014. National comprehensive cancer network. Survivorship: pain version 1.2014. *J. Natl. Compr. Canc. Netw.* 12 (4), 488–500.
- Di Cesare Mannelli, L., Pacini, A., Bonaccini, L., Zanardelli, M., Mello, T., Ghelardini, C., 2013. Morphologic features and glial activation in rat oxaliplatin-dependent neuropathic pain. *J. Pain* 14 (12), 1585–1600.
- Di Cesare Mannelli, L., Pacini, A., Matera, C., Zanardelli, M., Mello, T., De Amici, M., Dallanoco, C., Ghelardini, C., 2014. Involvement of $\alpha 7$ nAChR subtype in rat oxaliplatin-induced neuropathy: effects of selective activation. *Neuropharmacology* 79, 37–48.
- Di Cesare Mannelli, L., Corti, F., Micheli, L., Zanardelli, M., Ghelardini, C., 2015. Delay of morphine tolerance by palmitoylethanolamide. *Biomed. Res. Int.* 2015 (2015), 894732.
- Eidson, L.N., Murphy, A.Z., 2013. Persistent peripheral inflammation attenuates morphine-induced periaqueductal gray glial cell activation and analgesic tolerance in the male rat. *J. Pain* 14, 393–404.
- Ferrini, F., Trang, T., Mattioli, T.A., Laffray, S., Del'Guidice, T., Lorenzo, L.E., Castonguay, A., Doyon, N., Zhang, W., Godin, A.G., Mohr, D., Beggs, S., Vandal, K., Beaulieu, J.M., Cahill, C.M., Salter, M.W., De Koninck, Y., 2013. Morphine hyperalgesia gated through microglia-mediated disruption of neuronal Cl^- homeostasis. *Nat. Neurosci.* 16, 183–192.
- Fu, X., Zhu, Z.H., Wang, Y.Q., Wu, G.C., 2007. Regulation of proinflammatory cytokines gene expression by nociceptin/orphanin FQ in the spinal cord and the cultured astrocytes. *Neuroscience* 144, 275–285.
- Guerrini, R., Calò, G., Rizzi, A., Bianchi, C., Lazarus, L., Salvadori, S., Temussi, P., Regoli, D., 1997. Address and message sequences for the nociceptin receptor: a structure-activity study of nociceptin-(1-13)-peptide amide. *J. Med. Chem.* 40, 1789–1793.
- Hameed, H., Hameed, M., Christo, P.J., 2010. The effect of morphine on glial cells as a potential therapeutic target for pharmacological development of analgesic drugs. *Curr. Pain Headache Rep.* 14 (2), 96–104.
- Hao, J.X., Xu, I.S., Wiesenfeld-Hallin, Z., Xu, X.J., 1998. Anti-hyperalgesic and anti-allodynic effects of intrathecal nociceptin/orphanin FQ in rats after spinal cord injury, peripheral nerve injury and inflammation. *Pain* 76 (3), 385–393.
- Harrison, L.M., Kastin, A.J., Zadina, J.E., 1998. Opiate tolerance and dependence: receptors, G-proteins, and antioxiates. *Peptides* 19 (9), 1603–1630.
- Homberg, J.R., Mul, J.D., De Wit, E., Cuppen, E., 2009. Complete knockout of the nociceptin/orphanin FQ receptor in the rat does not induce compensatory changes in mu, delta and kappa opioid receptors. *Neuroscience* 163, 308–315.
- Horvath, R.J., DeLeo, J.A., 2009. Morphine enhances microglial migration through modulation of P2X4 receptor signaling. *J. Neurosci.* 29, 998–1005.
- Ji, R.R., 2010. Targeting microglial purinergic signaling to improve morphine analgesia. *Pain* 150, 377–378.
- Johansson, T., Elfverson, M., Zhou, Q., Nyberg, F., 2010. Allosteric modulation of the NMDA receptor by neurosteroids in rat brain and the impact of long term morphine administration. *Biochem. Biophys. Res. Commun.* 401, 504–508.
- Leighton, G.E., Rodriguez, R.E., Hill, R.G., Hughes, J., 1988. k-opioid agonist produce antinociception after i.v. and i.c.v. but not intrathecal administration in the rat. *Br. J. Pharmacol.* 93, 553–560.
- Lucas, G.A., Hoffmann, O., Alster, P., Wiesenfeld-Hallin, Z., 1999. Extracellular cholecystokinin levels in the rat spinal cord following chronic morphine exposure: an in vivo microdialysis study. *Brain Res.* 821 (1), 79–86.
- Lutfy, K., Hossain, S.M., Khaliq, I., Maidment, N.T., 2001. Orphanin FQ/nociceptin attenuates the development of morphine tolerance in rats. *Br. J. Pharmacol.* 134, 529–534.
- Malin, D.H., Lake, J.R., Hammond, M.V., Fowler, D.E., Rogillio, R.B., Brown, S.L., Sims, J.L., Leecraft, B.M., Yang, H.Y., 1990. FMRF-NH2-like mammalian octapeptide: possible role in opiate dependence and abstinence. *Peptides* 11 (5), 969–972.
- Mallimo, E.M., Kusnecov, A.W., 2013. The role of orphanin FQ/nociceptin in neuroplasticity: relationship to stress, anxiety and neuroinflammation. *Front. Cell. Neurosci.* 7, 173.
- Mamiya, T., Noda, Y., Ren, X., Nagai, T., Takeshima, H., Ukai, M., Nabeshima, T., 2001. Morphine tolerance and dependence in the nociceptin receptor knockout mice. *J. Neural Transm.* 108, 1349–1361.
- McGrath, J.C., Lilley, E., 2015. Implementing guidelines on reporting research using animals (ARRIVE etc.): new requirements for publication in BJP. *Br. J. Pharmacol.* 172 (13), 3189–3193.
- Meunier, J.C., Mollereau, C., Toll, L., Suaudeau, C., Moisand, C., Alvinerie, P., Butour, J.L., Guillemot, J.C., Ferrara, P., Monsarrat, B., 1995. Isolation and structure of the endogenous agonist of opioid receptor-like ORL1 receptor. *Nature* 377, 532–535.
- Micheli, L., Di Cesare Mannelli, L., Guerrini, L., Trapella, R., Zanardelli, C., Cicciocioppo, M., Rizzi, R., Ghelardini, A., Calò, G., 2015a. Acute and subchronic antinociceptive effects of nociceptin/orphanin FQ receptor agonists infused by intrathecal route in rats. *Eur. J. Pharmacol.* 754, 73–81.
- Micheli, L., Di Cesare Mannelli, L., Rizzi, A., Guerrini, R., Trapella, C., Calò, G., Ghelardini, C., 2015b. Intrathecal administration of nociceptin/orphanin FQ receptor agonists in rats: a strategy to relieve chemotherapy-induced neuropathic hypersensitivity. *Eur. J. Pharmacol.* 766, 155–162.
- Mika, J., Wawrzczak-Bargiela, A., Osikowicz, M., Makuch, W., Przewlocka, B., 2009. Attenuation of morphine tolerance by minocycline and pentoxifylline in naive and neuropathic mice. *Brain Behav. Immun.* 23, 75–84.
- Mika, J., 2008. Modulation of microglia can attenuate neuropathic pain symptoms and enhance morphine effectiveness. *Pharmacol. Rep.* 60, 297–307.
- Mogil, J.S., Grisel, J.E., Reinscheid, R.K., Civelli, O., Belknap, J.K., Grandy, D.K., 1996. Orphanin FQ is a functional anti-opioid peptide. *Neuroscience* 75 (2), 333–337.
- Morgan, M.M., Grisel, J.E., Robbins, C.S., Grandy, D.K., 1997. Antinociception mediated by the periaqueductal gray is attenuated by orphanin FQ. *Neuroreport* 8 (16), 3431–3434.
- Ozaki, S., Kawamoto, H., Itoh, Y., Miyaji, M., Iwasawa, Y., Ohta, H., 2000. A potent and highly selective nonpeptidyl nociceptin/orphanin FQ receptor (ORL1) antagonist: J-113397. *Eur. J. Pharmacol.* 387 (3), R17–R18.
- Raghavendra, V., Rutkowski, M.D., DeLeo, J.A., 2002. The role of spinal neuroimmune activation in morphine tolerance/hyperalgesia in neuropathic and sham-operated rats. *J. Neurosci.* 22, 9980–9989.
- Reinscheid, R.K., Nothacker, H.P., Boursion, A., Ardati, A., Henningsen, R.A., Bunzow, J.R., Grandy, D.K., Langen, H., Monsma, F.J., Jr, Civelli, O., 1995. Orphanin FQ: a neuropeptide that activates an opioidlike G protein-coupled receptor. *Science* 270, 792–794.
- Schröder, W., Lambert, D.G., Ko, M.C., Koch, T., 2014. Functional plasticity of the N/OFQ-NOP receptor system determines analgesic properties of NOP receptor agonists. *Br. J. Pharmacol.* 171 (16), 3777–3800.
- Self, D.W., Nestler, E.J., 1995. Molecular mechanisms of drug reinforcement and addiction. *Annu. Rev. Neurosci.* 18, 463–495.
- Song, P., Zhao, Z.Q., 2001. The involvement of glial cells in the development of morphine tolerance. *Neurosci. Res.* 39, 281–286.
- Takayama, N., Ueda, H., 2005. Morphine-induced overexpression of prepro-nociceptin/orphanin FQ in cultured astrocytes. *Peptides* 26, 2513–2517.
- Thorn, D.A., Zhang, Y., Li, J.X., 2016. Effects of the imidazoline I2 receptor agonist 2-BFI on the development of tolerance and behavioral/physical dependence to morphine in rats. *Br. J. Pharmacol.* 173 (8), 163–172.
- Tian, J.H., Xu, W., Fang, Y., Mogil, J.S., Grisel, J.E., Grandy, D.K., Han, J.S., 1997. Bidirectional modulatory effect of orphanin FQ on morphine-induced analgesia: antagonism in brain and potentiation in spinal cord of the rat. *Br. J. Pharmacol.* 120 (4), 676–680.
- Tomassoni, D., Amenta, F., Amantini, C., Farfariello, V., Di Cesare Mannelli, L., Nwanko, I.E., Marini, C., Tayebati, S.K., 2013. Brain activity of Thioctic acid enantiomers: in vitro and in vivo studies in an animal model of cerebrovascular injury. *Int. J. Mol. Sci.* 14 (3), 4580–4595.
- Ueda, H., Inoue, M., Takeshima, H., Iwasawa, Y., 2000. Enhanced spinal nociceptin receptor expression develops morphine tolerance and dependence. *J. Neurosci.* 20 (20), 7640–7647.
- Ueda, H., Yamaguchi, T., Tokuyama, S., Inoue, M., Nishi, M., Takeshima, H., 1997. Partial loss of tolerance liability to morphine analgesia in mice lacking the nociceptin receptor gene. *Neurosci. Lett.* 237, 136–138.
- Watkins, L.R., Hutchinson, M.R., Johnston, I.N., Maier, S.F., 2005. Glia: novel counter-regulators of opioid analgesia. *Trends Neurosci.* 28, 661–669.
- Xu, X.J., Hao, J.X., Wiesenfeld-Hallin, Z., 1996. Nociceptin or antinociceptin: potent spinal antinociceptive effect of orphanin FQ/nociceptin in the rat. *Neuroreport* 7 (13), 2092–2094.
- Yaksh, T.L., Rudy, T.A., 1976. Chronic catheterization of the spinal subarachnoid space. *Physiol. Behav.* 17, 1031–1036.
- Yamamoto, T., Nozaki-Taguchi, N., Kimura, S., 1997. Effects of intrathecally administered nociceptin, an opioid receptor-like1 (ORL1) receptor agonist, on the thermal hyperalgesia induced by carageenan injection into the rat paw. *Brain Res.* 754 (1–2), 329–332.
- Yuan, L., Han, Z., Chang, J.K., Han, J.S., 1999. Accelerated release and production of orphanin FQ in brain of chronic morphine tolerant rats. *Brain Res.* 826 (2), 330–334.
- Yuill, M.B., Zee, M.L., Marcus, D., Morgan, D.J., 2016. Tolerance to the antinociceptive and hypothermic effects of morphine is mediated by multiple isoforms of c-Jun N-terminal kinase. *Neuroreport* 27 (6), 392–396.
- Zeilhofer, H.U., Calò, G., 2003. Nociceptin/orphanin FQ and its receptor—potential targets for pain therapy? *J. Pharmacol. Exp. Ther.* 306, 423–429.
- Zhang, X., Bao, L., Li, S., 2015. Opioid receptor trafficking and interaction in nociceptors. *Br. J. Pharmacol.* 172, 364–374.