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THEMED ISSUE ARTICLE

Activation of peripheral NOP receptors reduces periorbital mechanical allodynia evoked by CGRP in mice

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Background and Purpose: Migraine is a neurovascular disorder largely mediated by calcitonin gene-related peptide (CGRP). This study explores the role of the nociceptin/orphanin FQ (N/OFQ)–N/OFQ receptor (NOP) system in CGRP-induced periorbital mechanical allodynia (PMA) in mice.

Experimental Approach: Male or female wild type (NOP(+/+)) and NOP receptor knockout (NOP(−/−)) mice and CD-1 mice were used. The brain penetrant, AT-403, and the peripherally restricted, UFP-112, NOP agonists were tested for PMA prevention. To identify a potential site of action at the cellular level, the ability of N/OFQ to signal at membrane and endosomal level in NOP-expressing HEK293 cells, and to inhibit the increase in cyclic adenosine monophosphate (cAMP) induced by CGRP in human Schwann cells (hSCs) was investigated.

Key Results: CGRP-induced PMA was comparable in NOP(+/+) and NOP(−/−) mice. AT-403 and UFP-112 equally reduced CGRP-evoked PMA in CD-1 mice. In NOP-expressing cells, activation of NOP resulted in the internalisation and movement of NOP away from the plasma membrane marker CAAX and to early endosomes marker Rab5a. N/OFQ stimulated $G_{\alpha i}$ recruitment to NOP at the plasma membrane and from the endosomal compartment. N/OFQ attenuated cAMP increase elicited by CGRP in hSCs.

Conclusions and Implications: The peripherally restricted NOP agonist showed efficacy similar to the brain-penetrant compound, indicating that peripheral NOP activation is sufficient to alleviate CGRP-evoked PMA. Despite NOP ability to halt $G\alpha_i$ recruitment and cAMP increase in cells, further studies are required to confirm that SCs are the cellular site where N/OFQ operates to attenuate the CGRP pro-migraine action.

Abbreviations: CLR/RAMP1, Calcitonin Receptor-Like Receptor/Receptor Activity-Modifying Protein-1; GTN, Glyceryl Trinitrate; hSC, human Schwann Cell; N/OFQ, Nociceptin/Orphanin FQ; NOP, Nociceptin/Orphanin FQ Peptide Receptor; PMA, Periorbital Mechanical Allodynia; SC, Schwann Cell.

KEYWORDS
AT-403, calcitonin gene-related peptide, migraine, nociceptin/orphanin FQ, NOP receptor, periorbital mechanical allodynia, UFP-112

1 | INTRODUCTION

Migraine is a neurovascular disorder that affects 10–15% of the adult population worldwide with a high individual and societal impact (Stewart et al., 2008; GBD 2019 Diseases and Injuries Collaborators, 2020; Steiner et al., 2020; Hautakangas et al., 2022). The recent introduction of drugs targeting the calcitonin gene-related peptide (**CGRP**) pathway has markedly expanded the therapeutic options for both acute (gepants) and preventive (gepants and anti-CGRP monoclonal antibodies) treatment of migraine, offering effective and well-tolerated options (Pellesi et al., 2024). However, a significant proportion of patients still do not respond adequately to these treatments, highlighting the need for additional therapeutic approaches (Pavelic et al., 2022; Zobdeh et al., 2021).

Nociceptin/orphanin FQ (**N/OFQ**) is the endogenous ligand of a G protein-coupled receptor now named **NOP receptor** (Meunier et al., 1995; Reinscheid et al., 1995). NOP is structurally similar to classical opioid receptors. NOP receptor activation results in cyclic adenosine monophosphate (cAMP) inhibition, closure of voltage-gated Ca^{2+} channels, and opening of inwardly rectifying K^{+} channels, leading to reduced neuronal excitability (Hawes et al., 2000). The NOP–N/OFQ system modulates several biological functions, including pain transmission (Lambert, 2008). As NOP receptor and N/OFQ peptide are highly expressed in rodent trigeminal ganglion and trigeminal nucleus caudalis and the NOP receptor in human trigeminal ganglion (Hou et al., 2003; Witta et al., 2004), a relationship between the N/OFQ/NOP system and migraine has been proposed (Hou et al., 2003; Kiguchi et al., 2020; Neal et al., 2001; Ozawa et al., 2015; Targowska-Duda et al., 2020; Witta et al., 2004; Xie et al., 1999). In rat trigeminal ganglion neurons, NOP activation suppresses the release of CGRP induced by various stimuli (Capuano et al., 2007, 2009). In vivo electrophysiological studies showed that N/OFQ inhibits excitatory responses in the trigeminal nucleus caudalis (Flores et al., 2001; Wang et al., 1996). Furthermore, in vivo, N/OFQ reduced neurogenic vasodilation in the *dura mater* (Bartsch et al., 2002) and the NOP agonist Ro 64-6198 attenuated allodynia in a mouse model of migraine induced by nitroglycerin (GTN) (Targowska-Duda et al., 2020).

Facial cutaneous allodynia is one component of a migraine attack (Lipton et al., 2008). In pre-clinical studies, periorbital mechanical allodynia (PMA) induced by local or systemically delivered pro-algesic substances has been extensively used to assess migraine-like pain, allowing the identification of potential therapeutic agents (De Logu et al., 2019; Romero-Reyes & Akerman, 2014). Robust preclinical and clinical evidence supports the role of CGRP in migraine mechanisms (Edvinsson et al., 2018). The pharmacokinetic properties of anti-CGRP medicines (i.e. monoclonal antibodies) (Johnson et al., 2019; Noseda et al., 2020) strongly suggest that CGRP acts at the peripheral level to elicit migraine pain. However, the precise peripheral cell types implicated in migraine pain signals remain uncertain. The hypothesis that CGRP released from C-fibre nerve terminals targets the calcitonin receptor-like receptor/receptor activity-modifying protein-1 (**CLR**/**RAMP1**) in Aδ-fibres nociceptors has been proposed (Edvinsson et al., 2019; Melo-Carrillo et al., 2017). However, more recently, it has been reported that CGRP sustains PMA in mice by activating the CLR/ RAMP1 complex in the cell membrane and in early endosomes of Schwann cells (SCs) surrounding cutaneous trigeminal fibres (De Logu et al., 2022).

In this study, we investigated the potential of targeting the NOP receptor as a therapeutic strategy in migraine. First, by using NOP knockout (NOP(−/−)) mice, we investigated whether the basal N/OFQergic activity attenuates the ability of CGRP to induce PMA in mice. Second, we evaluated the efficacy of two NOP selective agonists, the brain-penetrant **AT-403** (Ferrari et al., 2017) and the peripherally restricted **UFP-112** (Rizzi et al., 2007) to prevent CGRP-induced PMA. Third, in order to identify one possible cellular site of action of NOP agonists, we explored whether NOP inhibits CGRP signalling in endosomes in SCs.

What is already known?

- The NOP receptor is expressed in central and peripheral migraine-related structures, including trigeminal ganglion.
- A brain-penetrant NOP agonist prevents nitroglycerin-induced allodynia in mice.

What does this study add?

- Peripherally restricted and brain-penetrant NOP agonists equally reduce CGRP-induced allodynia.
- NOP activation inhibits CGRP-evoked cAMP increase in human Schwann cells.

What is the clinical significance?

- Peripheral NOP activation may be sufficient to counteract CGRP-related migraine pain.
- Peripherally acting NOP agonists may offer new therapeutic options for migraine management.

2 | METHODS

2.1 | Materials

α-rat CGRP (catalogue no. C0292) was purchased from Merck Life Science (Milan, Italy) and dissolved in phosphate buffered saline (PBS, catalogue no. ECB4053L, Euroclone, Milan, Italy). AT-403 was provided by Dr. Nurulain Zaveri (Astraea Therapeutics, Mountain View, CA, USA) and dissolved in 1% dimethyl sulfoxide (DMSO, catalogue no. D5879, Merck Life Science), 0.3% cyclodextrin (catalogue no. 332593, Merck Life Science) and distilled water. UFP-112 and N/OFQ were synthesised in house, as previously described (Arduin et al., 2007; Guerrini et al., 1997), and dissolved in PBS. CGRP (0.01–0.1 mg kg^{-1}), AT-403 (1–30 μg kg^{-1}), UFP-112 (1–100 pmol and 100 nmol), and their vehicles were injected intraperitoneally (i.p.) in a volume of 10 ml kg^{-1}.

2.2 | Animals

All the experimental procedures adopted in the in vivo studies comply with the European Directive 2010/63/EU on the protection of animals used for scientific purposes and Italian Legislative Decree no. 26 of 4 March 2014. These experiments have been approved by the Animal Welfare Body of the University of Ferrara and by the Ministry of Health (authorisation number 73/2021-PR). Animal studies are reported in compliance with the ARRIVE guidelines (Percie du Sert et al., 2020) and with the recommendations made by the *British Journal of Pharmacology* (Lilley et al., 2020). All experiments were conducted with mice bred and housed in the University of Ferrara's animal facility under specific pathogen-free conditions. All mice were housed in cages with individual ventilation, with a constant temperature of 21°C, 60% humidity and with a 12-h light/dark cycle. Food and water were provided ad libitum. CD-1 male and female mice, aged 2 to 4 months, were used. Details about the generation of NOP(−/−) and NOP(+/+) mice have been published previously (Briscini et al., 2002; Nishi et al., 1997); these mice have been backcrossed on to the CD-1 strain in our laboratories. NOP (+/+) and NOP(−/−) littermates were obtained by mating NOP (+/−) mice. All mice were genotyped using the polymerase chain reaction previously described (Holanda et al., 2019). All mice were used only once. At the end of the experiment, the animals were euthanized by CO_2 overdose, and death was confirmed by cervical dislocation.

2.3 | Periorbital mechanical allodynia

Experiments were conducted in plexiglass cylinders (diameter 11 cm, height 16 cm) capped at the top with a grid. Mice were habituated to the testing apparatus for 30 min 2 days prior to the experiment and again for 30 min right before the experiment began. PMA was measured using von Frey filaments (Ugo Basile srl, Varese, Italy) according to the up-and-down paradigm (Chaplan et al., 1994; Dixon, 1980). Measurements were taken in the periorbital region, situated caudal to the eyes and near the midline. Seven von Frey filaments varying in logarithmic increments of force (0.02, 0.04, 0.07, 0.16, 0.4, 1.0, and 1.4 g) were applied perpendicularly to the skin in the periorbital area, using just enough pressure to induce a slight buckling, and held for about 5 s to trigger a positive response. Stimulation began with the 0.16-g filament. A positive response was identified by one of the following criteria: the mouse stroking its face with its forelimb, withdrawing its head from the stimulus or shaking its head. The von Frey filament was promptly removed upon a positive response. If a response occurred, a filament with a smaller force (down) was used in the next test; conversely, if no response was observed, a heavier force filament (up) was used. Following the first breaking point, four additional measurements were taken for each mouse. If no breaking point was observed, four measurements were taken. The 50% mechanical withdrawal threshold (expressed in g) was calculated from these scores using the subsequent equation:

$$50\%\ \text{threshold},\ g = \frac{\left(10^{(Xf+k\delta)}\right)}{10{,}000}$$

The Dixon statistics table provides the *k* value; *Xf* is the last filament applied in the XO series. It can be inserted as either the handle number or log (target force), and the δ is defined as the mean difference (in log scale) between steps of the filament range (Chaplan et al., 1994). To induce migraine-like pain in mice, CGRP was injected i.p. at the dose of 0.1 mg kg^{-1}, as previously reported (Mason et al., 2017; Rea et al., 2018; Sturaro et al., 2023). To investigate differences in CGRP sensitivity in NOP(−/−) and NOP(+/+) mice, CGRP was tested at multiple doses in the range 0.01–0.1 mg kg^{-1}. The baseline measurement (time point 0) was assessed before the injection of any drug. Then, PMA was measured 0.5, 1 and 2 h after CGRP administration. NOP agonists were given 30 min before CGRP, and PMA was measured 0.5, 1 and 2 h after CGRP administration (1, 1.5 and 2.5 h after baseline detection, Figure 1). Experiments were performed between 9:00 AM and 2 PM.

2.4 | Rotarod test

The rotarod test was performed as previously described (Rizzi et al., 2016), using a constant speed device (Ugo Basile), setting a speed of 15 revolutions min^{-1}. Mice underwent an apparatus familiarisation session of 2 min the day before the experiment. On the day of the experiment, locomotor performance was calculated as time (seconds) spent on the rotarod. A cut-off time of 120 s was chosen. UFP-112 100 nmol was injected 30 min before the test (Figure 1). Experiments were performed between 9:00 AM and 2 PM.

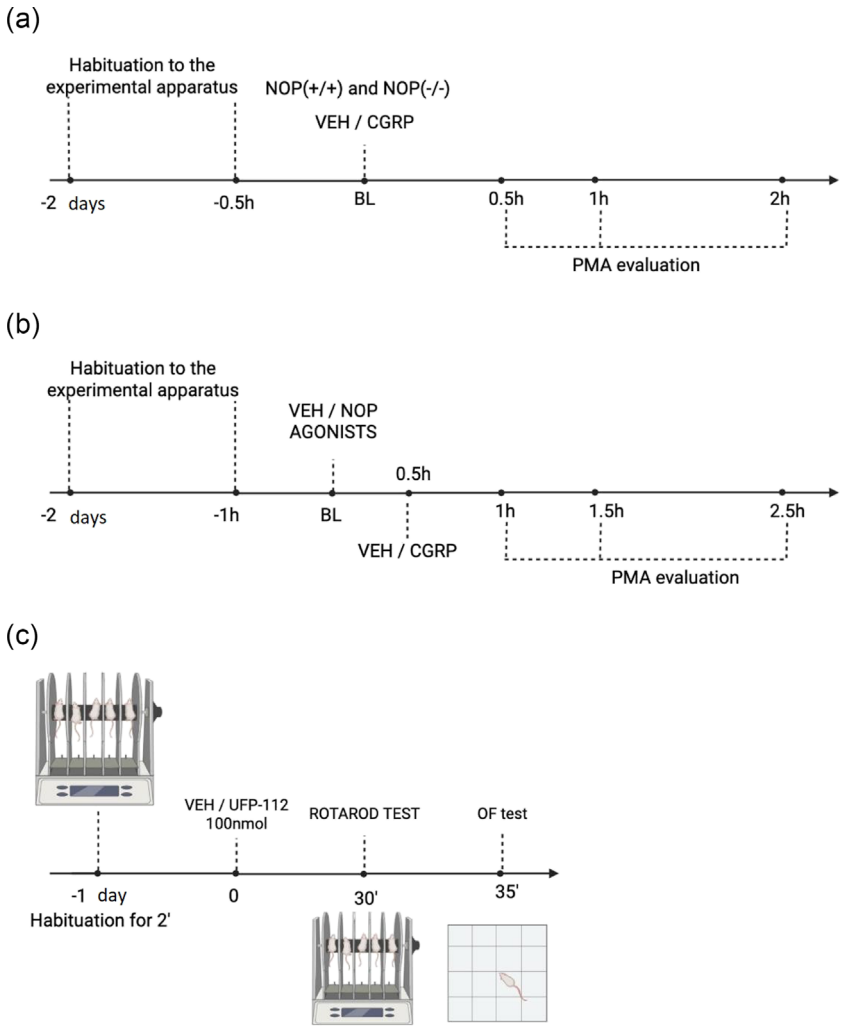

FIGURE 1 **Schematic representation of the in vivo experimental protocols used in this study.** (a) Schematic representation of the experimental timeline used to evaluate CGRP-induced Periorbital Mechanical Allodynia (PMA) across NOP(+/+) and NOP(−/−) mice. (b) Experimental design for assessing the effects of NOP agonists on CGRP-induced PMA. (c) Schematic representation of the experimental timeline used to evaluate the effects of UFP-112 on mice locomotor performance. BL, baseline; veh, vehicle.

2.5 | Open field test

The open field test was performed immediately after the rotarod assay on the same mice, using a protocol similar to that described by Azevedo Neto et al. (2021). Mice were positioned in square plastic cages (40 × 40 cm), one mouse per cage. Four mice were monitored in parallel. The test was performed under white light (1000 lx). The animal was placed in the centre of the arena and then allowed to explore the novel environment for 30 min. Distance travelled was recorded through the ANY-maze video tracking system (ANY-maze; Stoelting Co., Wood Dale, IL, USA; application version 4.52c Beta). UFP-112 100 nmol was given 30 min before the test (Figure 1). Experiments were performed between 9:00 AM and 2 PM.

2.6 | Cells

HEK293 cells (RRID CVCL_0045) were cultured and maintained in DMEM medium (catalogue no. 11965118, Thermo Fisher Scientific, Waltham, MA, USA) with 10% fetal bovine serum (catalogue no. 26140079, Thermo Fisher Scientific) and maintained at 37°C and 5% CO_2. Cells were passaged at 90% confluency. Primary human Schwann cells (hSCs) (catalogue no. P10351, Innoprot, Bizkaia, Spain) were cultured in Schwann cell medium (catalogue no. P60123, Innoprot) at 37°C in 5% CO_2 and 95% O_2 as previously reported (Titiz et al., 2024). After 12 passages, cells were discarded and replaced.

2.7 | Bioluminescence resonance energy transfer (BRET) and nanobioluminescence resonance energy transfer (nbBRET) assays

nbBRET experiments were performed as previously described (Edenson, 2020; Hegron et al., 2023; Latorre et al., 2022; Peach et al., 2021). NOP cDNA (cDNA.org #OPRL100000) was cloned into the pcDNA5-FRT vector with a C-terminal Renilla luciferase 8 (RLuc8) tag or a NanoBiT (NB) tag using the NEBuilder HiFi DNA assembly system (catalogue no. E5520S, New England BioLabs, Ipswich, MA, USA). The resulting plasmids were sequence-verified and amplified using chemicompetent DH5α bacteria. CAAX-GFP (plasma membrane

marker), Rab5a-GFP (early endosome marker), Venus-miniG$_{\alpha i}$ and Venus-miniG$_{\alpha s}$ were kindly provided by N. Lambert (Augusta University Medical College of Georgia, GA). CAAX-LgBiT (plasma membrane) and FYVE-LgBiT (early endosome) constructs were donated by the Alex Thompson lab (New York University). To assess NOP translocation from the plasma membrane to early endosomes, HEK293 cells were transfected in 10 cm dishes with NOP-RLuc8 (1 μg) and CAAX-GFP (3 μg) or Rab5a-GFP (3 μg). To evaluate the recruitment of G proteins at the plasma membrane or endosomes, HEK293 cells were transfected with NOP-NanoBiT (1 μg) and CAAX-LgBiT (2 μg) + Venus-miniG$_{\alpha i/\alpha s}$ (2 μg) or FYVE-LgBiT (2 μg) + Venus-miniG$_{\alpha i/\alpha s}$ (2 μg) using the PEI (catalogue no. 408727, Sigma-Aldrich, St. Louis, MO, USA) transfection method with a DNA:PEI ratio of 1:6 and incubated overnight.

After transfection, cells were plated in poly-D-lysine (catalogue no. P6407, Sigma-Aldrich) coated white-walled 96-well plates (30,000 cells per well) and incubated overnight in standard growth medium. Cells were then washed twice with Hanks' Balanced Salt Solution (HBSS, catalogue no. 14025092, Thermo Fisher Scientific) and incubated for 30 min before adding the luciferase substrates—coelenterazine H (catalogue no. 301, Nanolight Technology, Pinetop, AZ, USA; 2.5 μM) for RLuc8 or furimazine (catalogue no. N2572, Promega, Madison, WI, USA; 10 μM) for NanoLuc—for 15 min. BRET and nbBRET signals were measured for 30 min using the CLARIOStar plate reader (BMG Labtech, Cary, NC, USA) with specific filter settings (donor emission: 460 ± 40 nm; acceptor emission: 540 ± 25 nm). Baseline readings were recorded for 2.5–5 min before adding N/OFQ (10 nM to 1 μM). The BRET signal was calculated as the ratio of acceptor (GFP or Venus) emission to donor (RLuc8 or NanoLuc) emission. Data were normalised to baseline BRET/nbBRET values after subtraction of values from vehicle-treated wells.

2.8 | cAMP assay

hSCs were plated in 96-well poly-L-lysine-coated (catalogue no. P4707, Merck Life Science; 8.3 μM) black clear bottom plates (5×10^5 cells well^{-1}; PerkinElmer). To measure cAMP signalling in real time, cells were infected with a cADDIs BacMam virus (1.09×10^9 v g^{-1} ml^{-1}) encoding the green upward cAMP sensor (#U0200G, Montana Molecular, Bozeman, USA) for 24–48 h according to the manufacturer's instructions. On the day of experiments, hSCs were washed with HBSS (catalogue no. H1387, Merck Life Science) at pH 7.4 at 37°C. All the experiments were performed using a Zeiss Axio Observer 7 fluorescent microscope equipped with a fast filter wheel, a Digi-4 lens for recording excitations and an Ultra-fast Sutter Lambda DG4 Xenon excitation source (range 300–700 nm) (Zeiss, Milan, Italy). The fluorescent signals were recorded for approximately 6 min by microscopy ex/em 506/517 nm (filter set: FT 495, ex BP 470/40, em BP 525/50, interval 1 s). hSCs expressing the green upward cAMP sensor (catalogue no. U0200G, Montana Molecular) were stimulated with human CGRP (catalogue no. C0167, Merck Life Science; 1 μM), also in the presence of N/OFQ (10 nM to 10 μM) or vehicle (0.001% DMSO). The $\Delta F/F0$ ratio was calculated for each experiment, and the results were expressed as area under the curve (AUC).

2.9 | Statistical analysis

Data and statistical analysis complied with the recommendations of the *British Journal of Pharmacology* on experimental design and analysis in pharmacology (Curtis et al., 2025).

For the PMA assessment, the experimental sample sizes were determined a priori by performing a power analysis using GPower 3.1 software. In PMA experiments, power analysis was conducted based on the following criteria: effect size = 0.6, $\beta = 0.8$ and $\alpha = 0.05$. This resulted in the requirement of a minimum of 10 mice per group to achieve adequate statistical power. To evaluate whether a high dose of UFP-112 impairs the locomotor activity of mice (as measured by rotarod and open field tests), power analysis was performed with these inputs: effect size = 1.6, $\beta = 0.8$ and $\alpha = 0.05$. The analysis conducted under these criteria indicates that at least eight mice per group are required to maintain sufficient statistical power. The precise number of mice in each experimental group and the number of animals used for each experiment are detailed in the figure legends. A total of 454 animals were used in this study, and no animals were excluded. Mice were randomly assigned to pharmacological treatments via the randomizer.org platform. The experimenter was blinded to the treatments but not to the genotype of the animals. All data are presented as mean ± standard error of the mean (SEM) of *n* animals per group and were analysed using GraphPad Prism 9.03 software. For PMA experiments, the time course of the drug effect is illustrated with raw data and as the AUC. Statistical analyses were performed on the AUC data. Following confirmation of a normal distribution (using the Shapiro–Wilk test), data were analysed using analysis of variance (ANOVA) or the non-parametric Kruskal–Wallis test. Multiple comparisons were made using Tukey's or Dunn's post hoc tests only if the ANOVA or Kruskal–Wallis test yielded significant results (F achieved $P < 0.05$) and there was no significant variance inhomogeneity. The open field and rotarod experiments were analysed using Student's *t*-test. Differences were considered statistically significant at $P < 0.05$. The statistical tests used, and corresponding statistical values are noted in the figure legends. cAMP experiments were analysed using ANOVA. Multiple comparisons were made using Bonferroni's post hoc test. Post-hoc tests were run only if F achieved P<0.05 and there was no significant variance inhomogeneity. In BRET experiments, time-course data were expressed as AUC and subsequently normalised to the effect of N/OFQ 1000 nM. Normalised values were used to generate concentration–response curves. Concentration–response curves to agonists were analysed by a four-parameter logistic nonlinear regression model: Effect = baseline + (Emax − baseline)/(1 + 10^ ((log$_{EC50}$ − log[ligand]) slope)). pEC$_{50}$ values were expressed as mean and CL$_{95\%}$.

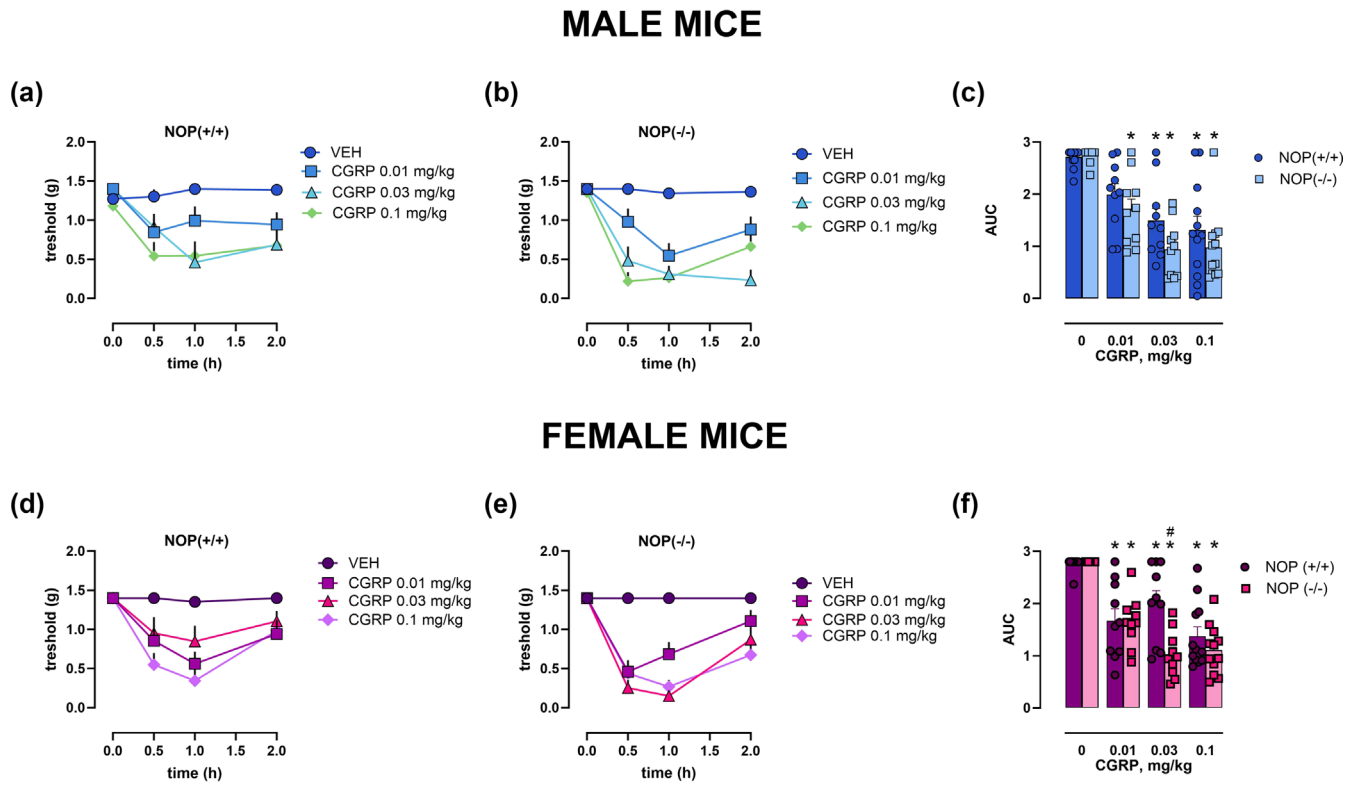

FIGURE 2 Effect of the i.p. administration of CGRP (0.01, 0.03 and 0.1 mg kg^{-1}) in NOP(+/+) and NOP(−/−) (a–c) male and (d–f) female mice. Threshold (g) was assessed 30 min, 1 h and 2 h following CGRP or vehicle administration. Figure 2a–e show the time course of the effect of CGRP, whereas Figure 2c–f show the same data expressed as AUC. Data are expressed as mean ± SEM, $n = 10$ mice per group with the exception of NOP(+/+) male and female, VEH $n = 12$ mice; NOP(+/+) and NOP(−/−) male and female, CGRP 0.1 mg kg^{-1} $n = 12$ mice; NOP (−/−) female, VEH $n = 11$ mice. Figure 2c: two-way ANOVA (treatment × genotype), followed by Tukey's multiple comparison test, revealed an effect of treatment ($F[3,78] = 30.39$), genotype ($F[1,78] = 4.710$). Figure 2f: two-way ANOVA (treatment × genotype), followed by Tukey's multiple comparison tests, revealed an effect of treatment ($F[3,79] = 40.89$), genotype ($F[1,79] = 8.065$) and their interaction ($F[3,79] = 4.409$). *$P < 0.05$ versus vehicle, #$P < 0.05$ versus NOP(+/+). Total mice used for this experiment: 173. AUC, area under curve; veh, vehicle.

2.10 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, and are permanently archived in the Concise Guide to PHARMACOLOGY 2023/2024 (Alexander et al., 2023).

3 | RESULTS

3.1 | Phenotype of NOP(+/+) and NOP (−/−) mice

CGRP (0.1 mg kg^{-1}, i.p.) induced PMA in both male and female NOP (+/+) and NOP(−/−) mice (Figure 2). Only when lower CGRP doses were tested, some marginally significant differences between NOP (+/+) and NOP(−/−) mice were recorded: CGRP 0.01 mg kg^{-1} induced PMA only in NOP(−/−), not in NOP(+/+) male mice (Figure 2c); CGRP 0.03 mg kg^{-1} induced higher PMA in NOP(−/−) than in NOP(+/+) female mice (Figure 2f). Data from NOP(+/+) and NOP(−/−) mice were analysed by two-way ANOVA (sex × treatment), and no significant effect of sex was detected.

3.2 | Effect of NOP agonists in CGRP-treated mice

The effects of NOP agonists were examined in CD-1 mice in vivo, against the robust PMA evoked by CGRP (0.1 mg kg^{-1} i.p.). The brain-penetrant NOP agonist AT-403 (30 μg kg^{-1}, i.p.) did not alter the baseline mechanical threshold in male and female mice (S1). AT-403 (30 min before CGRP) attenuated CGRP-induced PMA in a dose-dependent manner, both in male and female mice (Figure 3a–d). The peripherally restricted NOP agonist UFP-112 (100 pmol, i.p.) did not affect the baseline mechanical threshold in both male and female mice (S1). However, UFP-112 dose-dependently reduced CGRP-induced PMA, with a difference in the threshold active dose between male (0.1 pmol) and female mice (1 pmol) (Figure 3e–h). No sex-related differences were observed at any of the tested doses for either AT-403 or UFP-112. Of note, a dose of UFP-112 10,000 times higher than that able to reduce CGRP-evoked PMA (100 nmol) did not affect

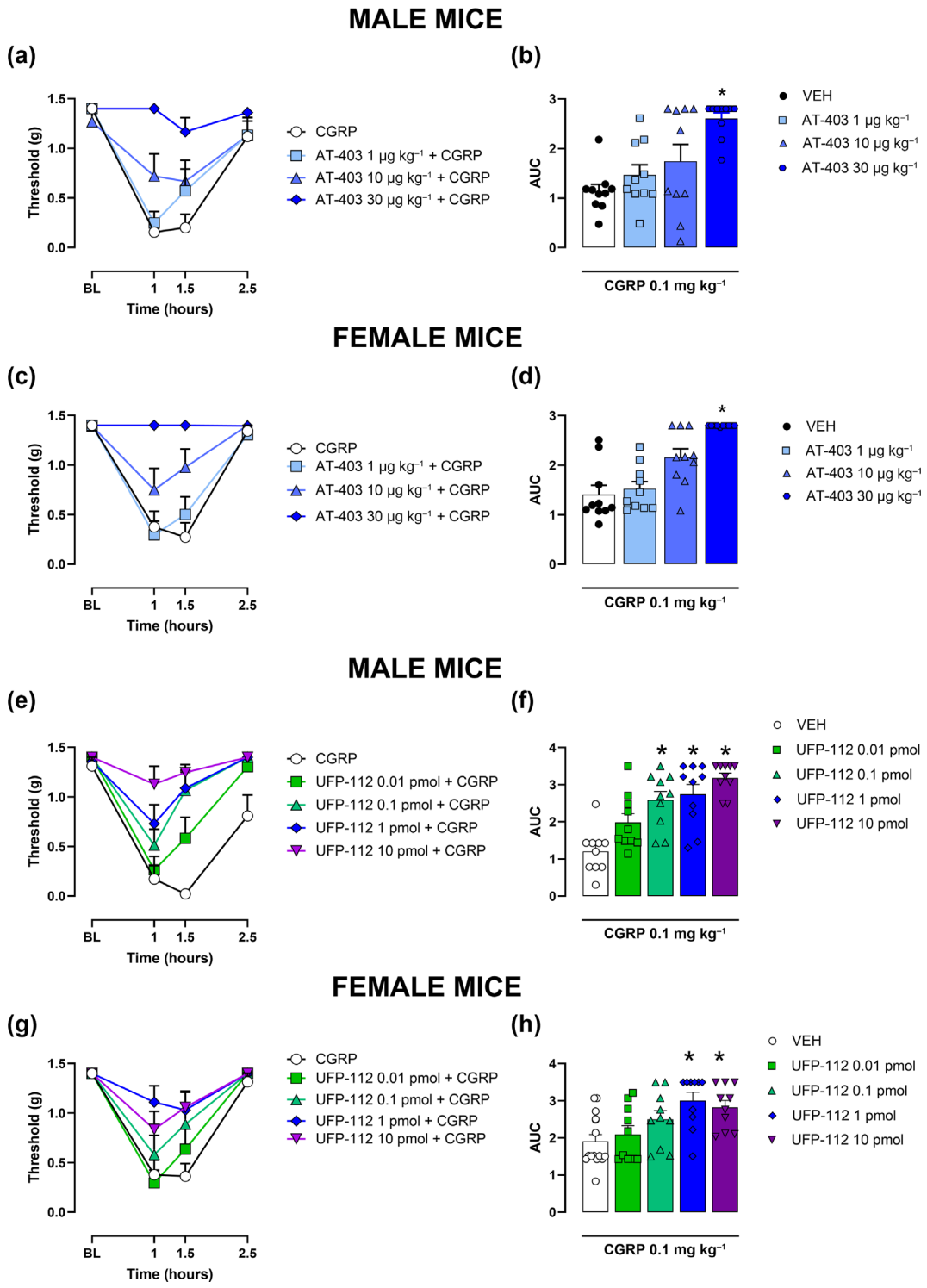

FIGURE 3 Effects of AT-403 and UFP-112 given i.p. 30 min before CGRP 0.1 mg kg^{-1} in CD-1 (a, b, e and f) male and (c, d, g and h) female mice. Sensitivity threshold (g) was assessed 1, 1.5 and 2 h following the administration of either vehicle, AT-403 or UFP-112. Left panels show the time course of the effect, right panels show the same data expressed as AUC. Data are expressed as mean ± SEM, $n = 10$ mice per group with the exception of Figure 3d–h, CGRP $n = 15$ mice per group. Kruskal–Wallis test followed by Dunn's test revealed an effect of AT-403 (Figure 3b: H3 = 15.71, Figure 3d: H3 = 23.69). Kruskal–Wallis followed by Dunn's test revealed an effect of UFP-112 (Figure 3f: H4 = 15.98, Figure 3h: H4 = 25.10). *$P < 0.05$ versus CGRP 0.1 mg kg^{-1} + vehicle. Total mice used for this experiment: 80 to test AT-403, 105 to test UFP-112. AUC, area under curve; BL, baseline; veh, vehicle.

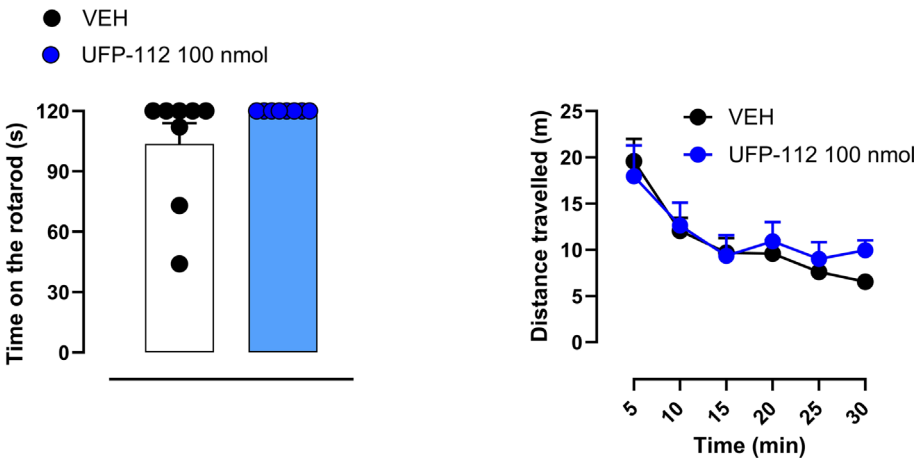

FIGURE 4 Effects of UFP-112 given i.p. in mice subjected to the (left) rotarod and (right) open field assays. Data are expressed as mean ± SEM, $n = 8$ mice per group. Total mice used for this experiment: 16. veh, vehicle.

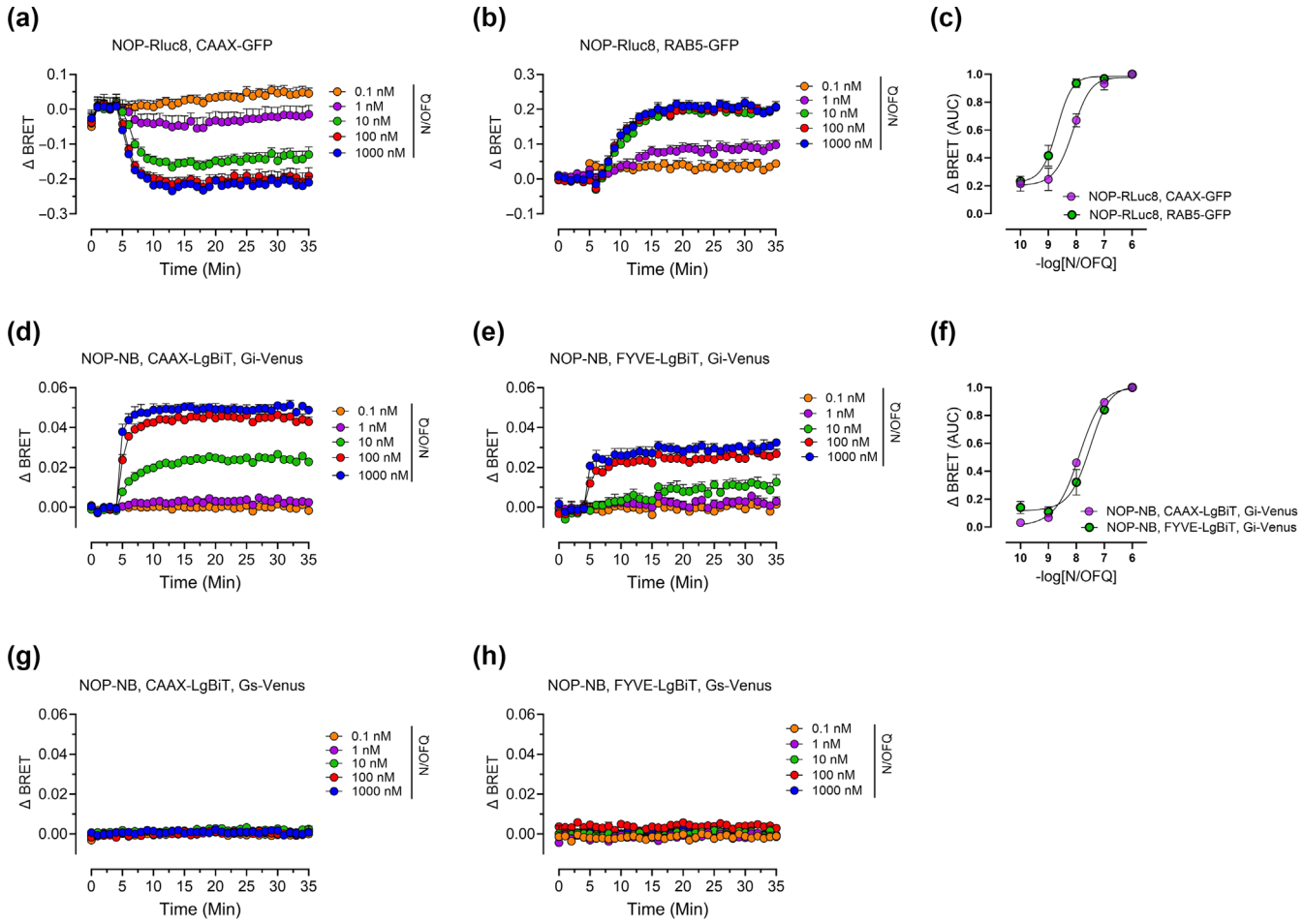

FIGURE 5 **BRET and nbBRET experiments in NOP-transfected HEK293 cells.** Effects of N/OFQ on NOP-CAAX (plasma membrane marker) and on NOP-RAB5 (endosome marker) interaction. (a and b) The time course of the effect, after subtraction of values from vehicle-treated wells. (c) The concentration–response curve to N/OFQ for data expressed as AUC and normalised as a fraction of N/OFQ 1000 nM effect. Effect of N/OFQ on NOP-mini$G_{\alpha i}$ interaction at (d) plasma membrane (time course of the effect) and at (e) endosomes (time course of the effect). (f) Data are shown as AUC and normalised as a fraction of N/OFQ 1000 nM effect. Effect of N/OFQ on NOP-mini$G_{\alpha s}$ interaction at (g) plasma membrane (time course of the effect) and at (h) endosomes (time course of the effect). Data are expressed as mean ± SEM, $n = 5$ separate experiments. AUC, area under curve.

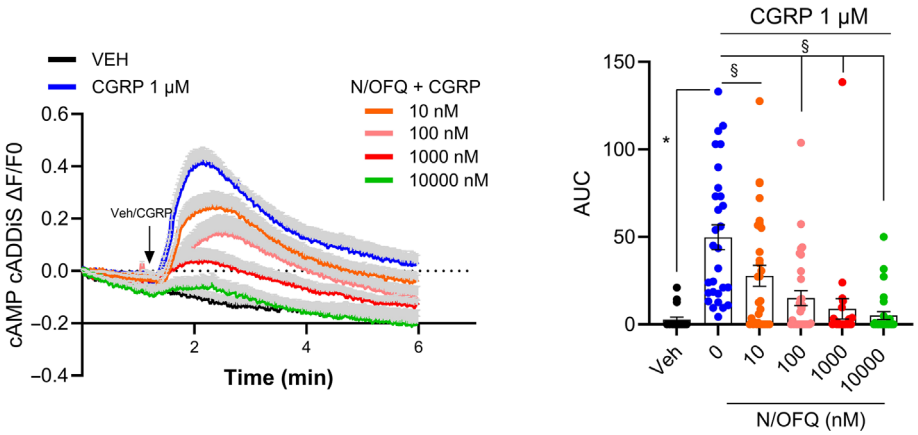

FIGURE 6 **Effects of CGRP and N/OFQ + CGRP on cAMP levels in human Schwann Cells (hSCs).** Representative traces (the coloured line is the average trace of each experiment, in grey the SEM) and cumulative data (AUC) of cAMP formation induced by CGRP in the presence of different concentration of N/OFQ. Data are the mean ± SEM of vehicle, $n = 18$; CGRP + N/OFQ 0 nM, $n = 29$; CGRP + N/OFQ 10 nM, $n = 30$; CGRP + N/OFQ 100 nM, $n = 31$; CGRP + N/OFQ 1000 nM, $n = 24$; CGRP + N/OFQ 10,000 nM, $n = 29$ cells. One-way ANOVA, Bonferroni correction. *$P < 0.05$ versus Veh, §$P < 0.05$ versus CGRP 1 μM. AUC, area under curve; veh, vehicle.

locomotor performance of mice on the rotarod as well as their spontaneous locomotor activity in the open field (Figure 4).

3.3 | In vitro effects of N/OFQ on NOP internalisation, Gi recruitment and cAMP

BRET assays showed that exposure of HEK293 cells expressing NOP to N/OFQ caused receptor translocation from cell membrane into endosomes. N/OFQ decreased in a concentration-dependent manner the interaction between NOP and the plasma membrane marker CAAX ($pEC_{50} = 8.11$ [7.58–8.64]), while increasing the interaction between NOP and the endosome marker RAB5a ($pEC_{50} = 8.72$ [8.23–9.14]) (Figure 5a–c). Additionally, nbBRET experiments showed that N/OFQ evokes mini$G_{\alpha i}$ recruitment both at plasma membrane and at endosomal level, with similar potency (membrane $pEC_{50} = 7.92$ [7.85–8.00], endosome $pEC_{50} = 7.53$ [7.39–7.68]) (Figure 5d–f). As expected, N/OFQ did not produce NOP-mini$G_{\alpha s}$ interaction (Figure 5g–h). Moreover, in hSCs, CGRP (1 μM) increased intracellular cAMP levels in a concentration-dependent manner. The natural NOP agonist N/OFQ concentration dependently attenuated this response with a pEC_{50} of ~8 (Figure 6).

4 | DISCUSSION

In this study, we observed no major differences between NOP(+/+) and NOP(−/−) mice in the PMA response, suggesting that the endogenous N/OFQ tone does not interfere with the mechanism underlying CGRP-evoked mechanical allodynia. This result does not align with previous data demonstrating exaggerated responses of NOP(−/−) animals in some pain models. Specifically, mice and rats lacking the functional NOP receptor exhibited increased sensitivity to formalin-induced pain in the hind paw (Depner et al., 2003; Rizzi et al., 2006, 2011) and within the trigeminal territory (Rizzi et al., 2017). These findings suggest that endogenous N/OFQ can be released in response to formalin but not CGRP. The difference may be explained by the fundamentally different proalgesic profiles of CGRP and formalin. While subcutaneous formalin induces a pronounced early non-evoked nociceptive response, characterised by licking and shaking (McNamara et al., 2007), subcutaneous CGRP fails to produce any non-evoked pain-like response (De Logu et al., 2019). Early nociceptor targeting may account for the different ability of the two substances to activate the endogenous N/OFQ–NOP receptor inhibitory pathway.

Previous evidence has proposed that NOP agonists attenuated proalgesic responses to migraine-producing substances. In particular, the NOP agonist Ro 64-6198 inhibited GTN-induced allodynia (Targowska-Duda et al., 2020), while the related NOP agonist Ro 65-6570 reduced pain behaviours induced by the injection of formalin in the orofacial region (Rizzi et al., 2017). The present study corroborates and extends these findings using the most important migraine pain mediator CGRP as a stimulus and two different NOP agonists: AT-403 and UFP-112. We previously showed that i.p. AT-403 produces analgesic effects in the formalin test in mice (Azevedo Neto et al., 2021). Notably, i.p. AT-403 (0.03 mg kg^{-1}) provided complete inhibition of CGRP-evoked PMA, without producing any visible reduction of locomotor activity, an effect produced by a much higher dose (1 mg kg^{-1}). As the inhibition of locomotor activity by AT-403 (1 mg kg^{-1}) was no longer evident in NOP(−/−) mice, indicating NOP-dependency of the agonist effect, the present data support a selective activation of the NOP receptor without confounding central influences from locomotion impairment. Present results corroborate previous findings (Targowska-Duda et al., 2020) demonstrating the antimigraine properties of systemically administered brain-penetrant NOP agonists.

However, results obtained with UFP-112 shift the focus to a peripheral site of action for the analgesic effects of NOP agonists. UFP-112 is a potent, selective and long-lasting NOP agonist designed by inserting several chemical modifications into the N/OFQ sequence to increase NOP receptor affinity/potency and/or reduce susceptibility to enzymatic degradation (Calò et al., 2011). Because of its peptide nature, high molecular weight and the presence of charged amino acids, UFP-112 does not cross the blood–brain barrier and can therefore be considered peripherally restricted (Calo' et al., 2011). Low doses (1–10 pmol) of i.p. UFP-112 completely prevent CGRP-induced PMA, suggesting that NOP activation in the periphery is sufficient to reduce CGRP-evoked PMA. Evidence that UFP-112 does not cross the blood–brain barrier comes from the observation that, while low doses (0.1 nmol) of UFP-112 given i.c.v. significantly reduced locomotor activity (Rizzi et al., 2007), a much higher systemic dose (100 nmol i.p., a dose 10,000 times greater than the dose effective in reducing PMA) failed to induce any motor impairment (Figure 4). The ability of UFP-112 to inhibit CGRP-evoked PMA supports the hypothesis that peripherally acting NOP agonists may be beneficial in migraine treatment. Central actions of brain penetrating NOP agonists, including SCH486757 or sunobinop, result in somnolence or hypnotic activity (Whiteside et al., 2024; Woodcock et al., 2010). The present findings, indicating a peripheral site of action, suggest a better efficacy and safety profile of peripherally restricting NOP agonists.

Given that migraine is predominantly a female condition and that distinct biological mechanisms may underlie the disease in each sex, and ensuing differences in drug responsiveness, it is crucial to conduct preclinical studies on novel migraine treatments in both males and females. However, in the present study, we did not find any significant sex-related differences, suggesting that NOP agonists may have comparable efficacy in both sexes. The NOP receptor is expressed both in the trigeminal nucleus caudalis and in the trigeminal ganglion (Neal et al., 1999; Targowska-Duda et al., 2020; Xie et al., 1999). Thus, NOP receptor activation might reduce migraine pain by acting at multiple sites in the central and peripheral nervous system. Results obtained with UFP-112 focus on NOP actions at the peripheral level. It could be hypothesised that NOP agonists reduce CGRP-induced PMA by attenuating CGRP's hypotensive effects, as vasodilatation might contribute to PMA. However, if vasodilatation were the mechanism underlying CGRP-evoked PMA, NOP agonists that reduce blood pressure (Giuliani et al., 1997; Madeddu et al., 1999; Rizzi et al., 2007) should enhance, rather than attenuate the pro-algesic response to CGRP. Recently, it has been shown that CGRP, released from trigeminal nerve endings, elicits PMA by activating the **CLR**/RAMP1 receptors expressed by SCs surrounding cutaneous trigeminal fibres (De Logu et al., 2022). In SCs, CGRP, binding to CLR/RAMP1, signals PMA by stimulating cAMP increases both at the plasma membrane and endosomes (De Logu et al., 2022). Building on these findings and considering that the ability to modulate pain signal from endosomes has already been reported for other opioid receptors (Fisher & von Zastrow, 2024; Jimenez-Vargas et al., 2020; Kunselman et al., 2021), we explored whether NOP receptor modulates endosomal signalling. Data in HEK293 cells indicate that N/OFQ promotes NOP receptor internalisation, from the cell surface to the endosomes, while sustaining Gi-dependent signals. Moreover, N/OFQ attenuated CGRP-evoked cAMP increase in human SCs. These results collectively suggest that the ability of NOP agonists to attenuate CGRP-evoked PMA in mice may depend on a physiological antagonism of NOP against CLR/RAMP1 signalling in membrane and endosomal compartments of SCs. Limitations of the present study are that in vitro studies were performed in human cell lines and not in primary cultures of mouse SCs and that no selective silencing procedure in SCs has been provided to support our hypothesis. Although our data provide evidence supporting stimulation of a peripheral NOP receptor, possibly in SCs, as a promising mechanism for migraine treatment, further studies with cell selective targeting of NOP and CLR/RAMP1 in SCs are necessary to provide final demonstration of the relevance of this mechanism. Future studies with the use of chronic migraine models, analysis of inflammatory markers, and profiling of receptor expression and signalling will help in the future to better define the mechanistic framework of the therapeutic potential of NOP agonists in migraine.

AUTHOR CONTRIBUTION

C. Sturaro: data curation; formal analysis; investigation (lead); methodology; visualisation and writing — original draft. **P. Pola**: investigation. **M. Argentieri**: investigation. **A. Frezza**: investigation. **M. Marini**: data curation; formal analysis; investigation and visualisation. **F. De Logu**: formal analysis; methodology; visualisation and writing — review and editing. **V. Albanese**: resources. **M. Soukupova**: methodology; writing — review and editing. **D. Malfacini**: conceptualisation; formal analysis; funding acquisition and writing — review and editing. **N. T. Zaveri**: resources; writing — review and editing. **R. Nassini**: conceptualisation; writing — review and editing. **D. Jensen**: conceptualisation; data curation; formal analysis; investigation; methodology; writing — review and editing. **G. Calò**: conceptualisation; funding acquisition; writing — original draft; writing — review and editing. **C. Ruzza**: conceptualisation; formal analysis; funding acquisition (lead); project administration; visualisation; writing — original draft (lead) and writing — review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the BJP guidelines for Design and Analysis, and Animal Experimentation, and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

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