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Investigation of the NOP receptor as a novel therapeutic target for migraine

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ABSTRACT

Background and Purpose: Migraine is one of the most prevalent neurological disorders worldwide, with a major impact on quality of life and public health. Its pathophysiology is complex and heterogeneous, leading to an unmet medical need for more effective and better-tolerated therapies. Classical treatments, are often limited by insufficient efficacy, adverse effects, and poor compliance. Even the recent advances with calcitonin gene-related peptide (CGRP)-targeting drugs leave a proportion of individuals insufficiently responsive. Therefore, the identification of novel therapeutic targets is of paramount importance. In this context, the Nociceptin/Orphanin FQ (N/OFQ)–NOP receptor system has emerged as a promising candidate, as several studies have linked it to migraine pathophysiology and suggested its potential as a druggable target. The general aim of this thesis was to investigate whether the NOP receptor represents a novel pharmacological target for anti-migraine therapy, and to generate and characterize new NOP receptor agonists as innovative anti-migraine drugs.

Experimental Approach: Male and female CD-1 wild-type (NOP(+/+)), and NOP receptor knockout (NOP(−/−)) mice were used. Migraine-related pain was modelled either by administration of CGRP 0.1 mg/kg, which induces periorbital mechanical allodynia (PMA), or by repeated restraint stress, which induces PMA and sensitization to a subthreshold dose of nitroglycerin (GTN, 0.1 mg/kg). The effects of NOP receptor activation were assessed using the brain-penetrant agonist AT-403 (1 - 30 μg/kg) and the peripherally restricted agonist UFP-112 (0.01 - 10 pmol). To identify a potential site of action at the cellular level, the ability of N/OFQ to signal at the 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. Finally, N/OFQ derivatives were generated through dimerization strategies. The in vitro activity of the compounds was evaluated using a NOP-G protein interaction BRET assay, a calcium mobilization assay, and the mouse vas deferens (mVD) bioassay The most interesting compounds were investigated in vivo in mice in the loss of the righting reflex (RR) test.

Key Results: CGRP-induced PMA was comparable in NOP(+/+) and NOP(-/-) mice. Similarly, no differences were recorded between NOP(+/+) and NOP(-/-) mice, neither in terms of stress susceptibility, nor in terms of sensitivity to a subthreshold

dose of GTN after stress sensitization. Both the brain-penetrant agonist AT-403 and the peripherally restricted agonist UFP-112 significantly reduced CGRP-evoked PMA with comparable efficacy. Similarly, in the stress–GTN model, AT-403 and UFP-112 prevented GTN-induced PMA in previously stressed mice and reversed stress-induced allodynia, including the persistent phenotype observed in aged animals. The equivalent effectiveness of AT-403 and UFP-112 across models highlights the prominent contribution of peripheral NOP receptors to the anti-migraine action of NOP agonists. In NOP-expressing cells, activation of NOP resulted in the internalization and movement of NOP away from the plasma membrane marker CAAX and to early endosomes marker Rab5a. N/OFQ stimulated Gαi recruitment to NOP at the plasma membrane and from the endosomal compartment. N/OFQ attenuated cAMP increase elicited by CGRP in hSCs. Finally, all dimeric N/OFQ derivatives behaved as full NOP agonists with different potency values. The most potent compound, **1h** (pEC_{50} > 9), was obtained by coupling two monomeric precursors via a Leu^{14}-to-Cys substitution. In vivo, **1h** demonstrated threefold greater potency than N/OFQ in eliciting loss of the RR in mice and produced a long-lasting effect monitored for up to 7 hours, supporting dimerization as a viable approach to developing long-acting peptide-based NOP ligands.

Conclusion and Implications: In conclusion, this thesis demonstrates that NOP agonists, particularly peripherally restricted compounds, may represent new therapeutic options for migraine. Among them, compound **1h**, characterized by high potency and long-lasting action, shows potential as a candidate drug. Although the mechanisms underlying the anti-migraine effects of NOP agonists are not yet fully clarified, the hypothesis of a functional antagonism between NOP and CGRP receptors is intriguing and warrants further investigation.

TABLE OF CONTENTS

1 INTRODUCTION 1

1.1 MIGRAINE 1

1.1.2 Migraine pathophysiology 2

1.1.3 Pharmacology of migraine: current treatments 5

1.1.4 Animal models of migraine 7

1.2 THE NOCICEPTIN/ORPHANIN FQ – NOP RECEPTOR SYSTEM 11

1.3.1 NOP receptor 11

1.3.2 Nociceptin/orphanin FQ 14

1.3.3 Biological activities of Nociceptin/Orphanin FQ – NOP receptor system 15

1.2.4 N/OFQ-NOP receptor system and migraine 18

AIM OF THE STUDY 21

2 RESEARCH WORK 22

2.1 ***Activation of peripheral NOP receptors reduces periorbital mechanical allodynia evoked by CGRP in mice*** 22

2.2 NOP receptor activation dampens stress-induced migraine-like behaviors in mice 36

2.3 Synthesis and Pharmacological Characterization of Nociceptin/Orphanin FQ Dimeric Ligands 59

GENERAL CONCLUSIONS 71

BIBLIOGRAPHY 74

ABBREVIATION LIST

- AMPA - α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
- APN - aminopeptidase N
- ATP - adenosine triphosphate
- cAMP -adenosine 3',5'-cyclic monophosphate
- CGRP - calcitonin gene-related peptide
- CNS - central nervous system
- CSD - cortical spreading depression
- DHE - dihydroergotamine
- DRG - dorsal root ganglia
- ECL - extracellular loop
- EEG - electroencephalogram
- EGFP - Enhanced Green Fluorescent Protein
- EP - endopeptidase
- FHM1 - familial hemiplegic migraine type 1
- Gln - glutamine
- GPCR - G protein-coupled receptor
- GRK - G-protein Receptor Kinase
- GTN - glyceryl trinitrate
- HPA - hypothalamic–pituitary–adrenal
- hSC - human Schwann cell
- i.c.v. - intracerebroventricularl
- ICHD - Internation Classification of Headache Disorders
- ICL - intracellular loop
- JNK - c-Jun N-terminal kinase
- mAb - monoclonal antibody
- MOH - medication overuse headache
- mVD - mouse vas deferens
- N/OFQ - Nociceptin/Orphanin FQ
- NMDA - N-methyl-D-aspartate receptor
- NO - nitric oxide
- NOP - Nociceptin Opioid Receptor
- NSAIDs - Nonsteroidal Anti-Inflammatory Drugs
- ORL1 - opioid receptor like 1
- PACAP - pituitary adenylate cyclase-activating peptide
- PAG - periaqueductal grey
- Phe - phenylalanine
- PKA - protein kinase A
- PKCγ - protein kinase C gamma
- PMA - periorbital mechanical allodynia
- ppN/OFQ - prepronociceptin
- REM - rapid eye movement
- ROS - reactive oxygen species
- RR - righting reflex
- RVM - rostral ventromedial medulla
- SA - specific aim
- SBDD - structure-based drug discovery
- SIA - stress-induced analgesia
- SIH - stress-induced hyperalgesia

- SNL - spinal nerve ligation
- TM - transmembrane helix
- VIP - vasoactive intestinal peptide

1 INTRODUCTION

1.1 MIGRAINE

Among the most prevalent neurological disorders that affect the global population, headaches stand out due to their spread and significant impairment on quality of life. Headache disorders are classified and diagnosed using the International Classification of Headache Disorders (ICHD), published by the International Headache Society (IHS) which serves as a gold standard text. Headaches are commonly divided into primary headaches, which lack an underlying cause for the symptomatology, and secondary headaches, which are typically symptoms of other conditions [1].

Migraines are a type of primary headaches affecting approximately 10-15% of the global population, with a prevalence that is disproportionately high in women (3:1 ratio) [2], [3]. Migraines are the second leading cause of years lived with disability according to the 2016 Global Burden of Disease Study. Migraine elevated prevalence is higher in developed nations compared to developing ones. The distribution of migraine prevalence is affected by age, with a rise throughout childhood and adolescence, peaking at middle age and gradually declining with aging [4]. These conditions impact individuals, families, workplaces, and healthcare systems. Chronic migraine, in particular, involves a severely reduced quality of life and presents harder daily challenges compared to episodic migraineurs [5]. Chronic migraine is also associated with high direct and indirect costs.

Migraines are characterized by a recurrent nature, complex and diverse pathophysiology, and symptomatology. A primary distinction is made between migraines with or without aura, a neurological symptom that precedes the headache phase. Migraine without aura is the most common types, characterized by moderate-to-severe, usually unilateral, throbbing pain, along with co-symptoms such as nausea, and increased sensitivity to light and sounds. The attacks can last from 4 to 72 h if untreated [6]. Migraine with aura, instead, is preceded by visual disturbances, such as blurred vision, flashing lights, blind spots, zigzag patterns, and tingling in the face or in the body. These symptoms last from 5 to 60 minutes and typically resolve before the pain phase onset [6].

The triggers for migraine can be very diverse, including hormonal changes in women, stress, lack of sleep, strong illumination, intense odors, beverages like

coffee and alcohol [7]. The recurrence of headache attacks can lead to chronic migraine, defined as at least 15 headaches per month, of which with 8 or more have migraine features.

Numerous studies have demonstrated that both acute and chronic stress are powerful triggers for migraine attacks [8]. The increased susceptibility of some patients to developing headaches and migraines suggests a maladaptive mechanism within nociceptive circuits, potentially leading to greater frequency or intensity of attacks [9], [10]. The underlying mechanism is complex and not fully understood. Among the plausible hypotheses, evidence indicates that stress lowers the threshold for cortical spreading depression (CSD) [11], [12], enhances nitric oxide synthesis with associated pro-inflammatory activity, and modulates hypothalamic signaling in the trigeminal nucleus caudalis, resulting in altered sensitivity of trigeminal nociceptors [13]. Interestingly, migraine attacks often do not occur during the stressful event itself but rather after its resolution [14]. The period of greatest vulnerability is typically 6 to 18 hours following stress cessation. Moreover, several consecutive days of intense stress are more likely to precipitate attacks than a single day of stress exposure [8]. In humans, anxiety and depression, frequently associated with prolonged stress, have also been observed to increase the frequency and severity of migraine episodes [15]. The frequent comorbidity with depression is significant. Results from an observational cohort showed that patients experiencing episodic migraine with depression are at a higher risk of chronic migraine onset. Both genetic predispositions and environmental factors can have an influence on this comorbidity [16].

1.1.2 Migraine pathophysiology

Activation of the trigeminovascular system (Figure 1), particularly its nociceptors innervating the dura mater, by mechanical, chemical or electrical stimuli results in migraine-like symptoms, including pain, nausea and photophobia [17].

The trigeminal nerve is the fifth cranial nerve involved in the innervation of the face and important brain vessels. As a mixed nerve, the trigeminal branches incorporate fibers dedicated to proprioception, pressure, vibration and nociception as well as efferent motor fibers. The sensory fibers innervating cerebral vessels and meninges are predominantly nociceptive, while autonomic sympathetic and parasympathetic efferents also reach meningeal vessels. Afferent fibers (primary neurons) converge

in the trigeminal ganglion and in the trigeminal root. Most of the nociceptive fibers terminate in the spinal trigeminal nucleus (trigeminal nucleus caudalis), which process pain and thermal information from the facial and cranial tissues. From there, secondary neurons project to the thalamus through the trigeminothalamic tracts. This relay regulates homeostasis, integrates pain signals, and connects to the limbic system (amygdala, nucleus accumbens), linking visceral pain and emotions [18].
In the thalamus, third-order neurons project to various areas of the somatosensory cortex. Pain and noxious thermal signals reach conscious perception via thalamocortical processing; precise localization and the perception of intensity and quality require further integration in the primary and secondary somatosensory cortices (S1 and S2) [18].

Trigeminal neurons can conduct action potentials orthodromically (toward the central nervous system (CNS)) and antidromically (toward the peripheral branches of the trigeminal divisions). The latter mediates the peripheral release of inflammatory neuropeptides typically involved in migraine onset. The axon terminals of these nociceptors contain vasoactive peptides such as calcitonin gene related peptide (CGRP), substance P, neurokinin A, and pituitary adenylate cyclase-activating peptide (PACAP), which promote vasodilation of dural vessels [19]. This process constitutes neurogenic inflammation, characterized by vasodilation, plasma extravasation, mast cells degranulation and white blood cells recruitment. If persistent, it can lead to a peripheral and/or central sensitization which lowers the threshold for spontaneous neuronal activity and hypersensitizes the system to cephalic and extracephalic stimuli, leading to aberrant responses: allodynia and hyperalgesia [20], [21]. Recently, a fundamental interplay was reported between trigeminal neurons and Schwann cells in CGRP-related sensitization of primary neurons. In particular, the CGRP receptor complex (CLR-RAMP1) can internalize and signal from inside the endosome in Schwann cells, with increase in adenosine 3’,5’-cyclic monophosphate (cAMP), activation of protein kinase A (PKA), and ultimately generation of nitric oxide (NO) and reactive oxygen species (ROS). NO and ROS excite adjacent C- or Aδ- fibers, causing periorbital allodynia in mice [22]. Interestingly, several authors propose CSD as a trigger for neurogenic meningeal inflammation, subsequently activating the trigeminal system [23]–[26]. Specifically, CSD is associated with elevated levels of adenosine triphosphate (ATP), glutamate, and potassium, which diffuse out of the cortex toward the pia/meninges and stimulate meningeal nociceptors [26], [27]. Moreover, during CSD, cortical neurons

may promote the synthesis of prostaglandins and cytokines in glial cells, leading to activation of trigeminal receptors located in the pia mater and pial arteries. This results in orthodromic activation of the trigeminal nerve and transmission of nociceptive signals to the CNS [27]. Nonetheless, the precise nature and mechanisms underlying the episodic activation of the trigeminovascular pathway in migraine remain poorly understood.

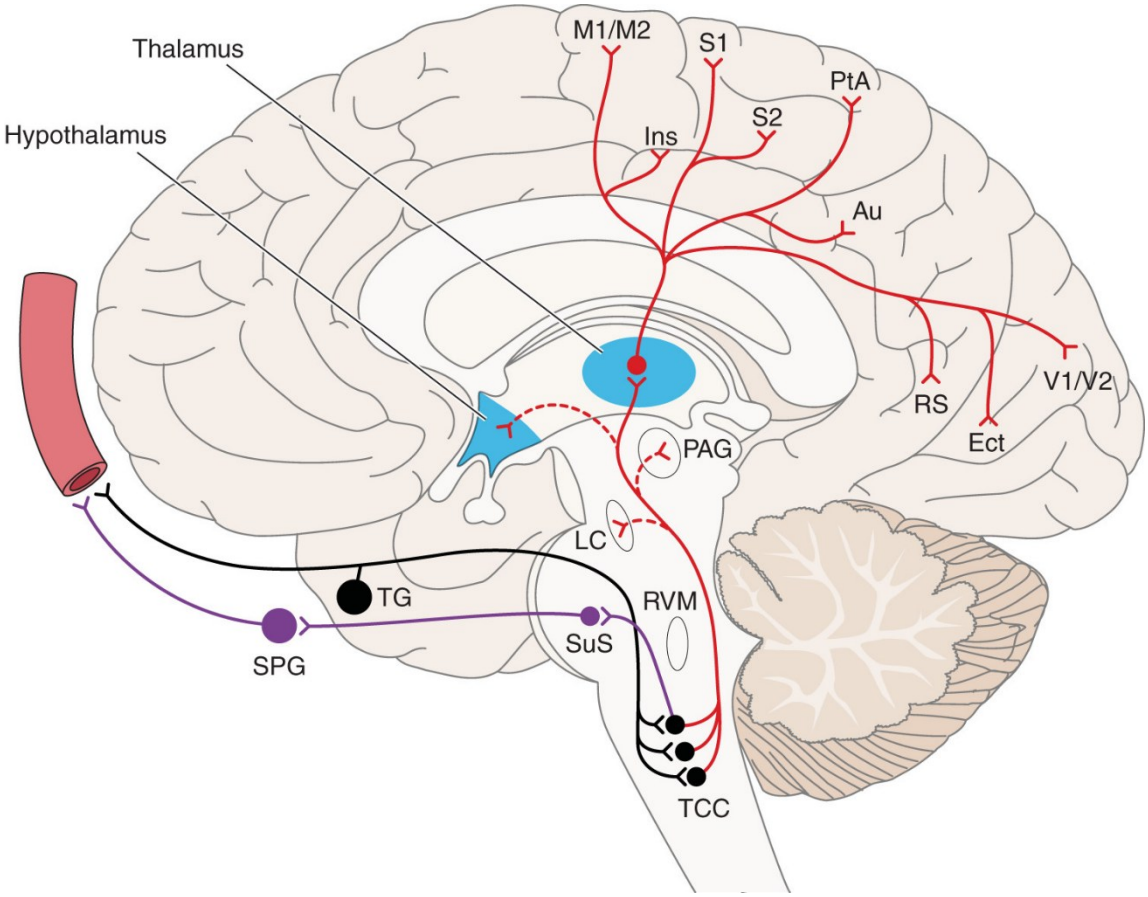

Figure 1: Anatomy of the trigeminovascular system. The trigeminal ganglion (TG) gives rise to trigeminal primary afferents which synapse on intra- and extracranial structures (blood vessels) as well as the spinal cord trigeminocervical complex (TCC). Second-order neurons from the TCC ascend in the trigeminothalamic tract synapsing on third-order thalamocortical neurons. The third-order thalamocortical neurons in turn synapse on a diffuse network of cortical regions including somatosensory (S1/S2) cortices.
Locus coeruleus (LC); superior salivatory nucleus (SuS); sphenopalantine ganglion (SPG); secondary motor (M1/M2) and visual (V1/V2) corteces; Ins, insula; PtA, parietal association; RS, retrosplenial; Au, auditory; Ect, ectorhinal; RVM, rostral ventromedial medulla. Taken from [17].

1.1.3 Pharmacology of migraine: current treatments

Current pharmacological approaches to migraine management can be broadly divided into two major categories: specific pharmacological treatments targeting the trigeminovascular system and CGRP pathway, and non-specific pharmacological treatments targeting the symptomatology [28]. The acute therapy aims to achieve rapid symptom relief, minimize functional disability, and reduce the duration and severity of migraine attacks. According to the US Headache Consortium, the goals of acute migraine treatment include: i) prompt and consistent crisis management without recurrence; ii) restoration of patient well-being while minimizing the need for additional medications; iii) reduction in symptomatic drug use; iv) cost-effectiveness; and v) minimal or no adverse effects. Preventive therapy is considered effective if it achieves a ≥50% reduction in attack frequency within three months.

Specific Pharmacological Treatments

Drugs Targeting the 5-HT System (Ergot Alkaloids, Triptans, and Ditans) – Ergot alkaloids, such as ergotamine (approved in 1976) and dihydroergotamine (DHE), have been used for decades to manage acute migraine attacks, with or without aura, and cluster headaches. DHE interacts with serotonergic, dopaminergic, and α-adrenergic receptors [29]. Its use is generally reserved for patients unresponsive to triptans. A novel nasal powder formulation of DHE (STS101) has undergone Phase 3 clinical trials as an acute anti-migraine agent (ASCEND, NCT04406649) [28].

Triptans have served as first-line acute migraine therapy for nearly 25 years. As serotonin analogues, they stimulate 5-$HT_{1B/1D}$ receptors in cranial vessels and nerve terminals, inhibiting the release of CGRP and substance P, and producing vasoconstriction. Sumatriptan was the first in this class, designed to retain ergot-like efficacy with fewer vascular side effects. Triptans are well tolerated in patients without vascular disease or uncontrolled hypertension, but overuse may cause medication-overuse headache (MOH). Crucially, they are contraindicated in patients with ischemic cardiovascular disease, cerebrovascular disease, peripheral vascular disease, or uncontrolled hypertension [28]. They are generally avoided during pregnancy; if needed, sumatriptan has the most reassuring data. Food and Drug Administration (FDA)-approved triptans include Zolmitriptan (1997) and Rizatriptan (1998), both effective for menstrual migraine. Ongoing research focuses on developing acute treatments targeting trigeminal pathways without engaging vasoactive 5-HT receptors [30].

The development of selective 5-HT_{1F} agonists (ditans) represents this shift. Lasmiditan, a selective 5-HT_{1F} agonist approved in 2019, differs structurally from triptans by replacing the indole core with a pyridine-piperidine scaffold. Unlike triptans, which carry cardiovascular risk due to 5-$HT_{1B/1D}$ mediated vasoconstriction, ditans act selectively on 5-HT_{1F} receptors at the neuronal level, without vasoconstriction [31]. Preclinical data show that Lasmiditan inhibits CGRP release from trigeminal neurons and prevents plasma protein extravasation in rodents [32]. Lasmiditan is generally suitable for patients with cardiovascular risk factors. Clinically, CNS adverse effects (dizziness, somnolence) are common and driving is restricted for 8 hours after dosing. Its safety and efficacy are currently under Phase 3 evaluation in pediatric populations (NCT04396574).

Drugs Targeting the CGRP System (gepants and monoclonal antibodies*)* – The development of CGRP-targeted therapies represents the most significant paradigm shift in migraine pharmacology in the last three decades. Gepants are small-molecule CGRP receptor antagonists, effective in acute and preventive treatment and particularly valuable in patients with triptans contraindications or inadequate response. Three gepants have received FDA approval for acute migraine treatment: Ubrogepant (2019), the first oral CGRP receptor antagonist, developed by Allergan (Ubrelvy™), Rimegepant (2020, also European Medicines Agency (EMA)-approved in 2022), also available for prevention, and Zavegepant as nasal spray (Zavzpret by Pfizer, 2023). Common side effects include mild nausea, somnolence, and dry mouth [33]. These oral antagonists are suitable as first-line treatments in patients at cardiovascular risk and as second-line therapy following triptan failure [34]. Monoclonal antibodies (mAbs) targeting CGRP or its receptor represent the first migraine-specific preventive drugs explicitly developed for prophylaxis [35]. Four CGRP mAbs are approved: Erenumab (targets the CGRP receptor), Galcanezumab, Fremanezumab, and Eptinezumab (target CGRP directly) [36]. Compared to gepants, they offer greater specificity, longer half-life, and fewer drug interactions but require parenteral administration [37]. While the American Headache Society recommends CGRP mAbs after failure of two oral preventives, the European Headache Federation now supports their first-line use [38]. Systematic reviews confirm their favorable benefit-risk profile compared to older prophylactics [39]. Both classes show minimal cardiovascular risks, though erenumab has been associated with hypertension warnings [40]. Common adverse effects include nausea, fatigue, constipation, and upper respiratory infections.

Despite the clinical success and revolutionary impact of CGRP-pathway drugs, only 69-79% of patients experienced a reduction in migraine attack frequency by 50% [41]. Furthermore, potential long-term risks associated with chronic CGRP blockade remain under investigation. These unmet needs underscore the continued necessity for identifying and validating alternative, non-CGRP pharmacological targets for migraine treatment.

Non-Specific Pharmacological Treatments

Nonsteroidal Anti-Inflammatory Drugs (NSAIDs) – NSAIDs are widely used for pain, inflammation, and fever. They act primarily through inhibition of cyclooxygenase (COX)-1 and/or COX-2 enzymes, reducing prostanoid synthesis and thereby dampening inflammation and pain signaling [28].

Acetaminophen (Paracetamol) - Acetaminophen's mechanism remains partly unclear, but it likely involves prostaglandin synthesis inhibition [42]. It shows good efficacy in migraine, alone or in combination with aspirin and caffeine [43]. Opioid containing combinations should be avoided because of MOH risk [44].

Dopamine Antagonists (Antiemetics) – Agents such as metoclopramide, chlorpromazine, and prochlorperazine alleviate both migraine pain and nausea. Side effects include sedation, hypotension, blurred vision, arrhythmias, and extrapyramidal symptoms, although these are uncommon with intermittent oral dosing [28].

Opioids – Opioids act on the trigeminovascular system to modulate pain but lack vasoconstrictive or anti-inflammatory effects. Due to their high abuse potential and risk of MOH, their use should be reserved for refractory cases and only after careful risk assessment [28].

Other non-specific preventives include beta-blockers (e.g., propranolol, metoprolol), calcium channel blockers, antiepileptics (e.g., topiramate and valproate), and OnabotulinumtoxinA [31].

1.1.4 Animal models of migraine

Animal models are essential tools for investigating disease mechanisms and testing potential therapeutic approaches. To be scientifically relevant, an animal model must possess external validity, i.e. the ability to generalize experimental findings to

other populations, including humans. The ultimate goal of using animal models is translational: results obtained in the laboratory should inform and guide clinical applications in patients. The external validity of an animal model is defined by three interrelated criteria: i) *construct validity* – refers to the degree to which the biological mechanisms underlying the animal model correspond to those involved in the human disease; ii) *face validity* – indicates that the model reproduces key clinical manifestations observed in patients; iii) and *predictive validity* – assesses whether the model responds appropriately to treatments known to be effective , or ineffective, in humans.

Despite these criteria, several factors limit the translational power of animal models. Pain studies are often performed on young, healthy, genetically uniform male animals, which contrasts with the clinical reality where pain predominantly affects women and older adults with comorbidities and diverse genetic backgrounds. Moreover, animal models cannot fully capture the multidimensional nature of human pain, which is shaped by psychological, social, and environmental influences. They also fail to replicate the chronic, progressive course of human diseases that evolve over years rather than weeks. Finally, because pain cannot be directly measured in animals, researchers must rely on surrogate behavioral indicators, such as withdrawal thresholds or facial grimace scales, whereas in humans, pain is assessed through self-reporting [45].

Several validated rodent models of migraine have been developed to study the disorder's pathophysiology and to evaluate innovative pharmacological targets. These models aim to reproduce the neurobiological, behavioral, and pharmacological features of human migraine attacks, providing insight into disease mechanisms and facilitating drug discovery. However, like other pain models, migraine models face limitations in fully reflecting the clinical complexity of the disease. Typically, mice and rats are used for modelling migraine *in vivo*. In each model it is possible to identify a trigger, a stimulus of various nature applied to induce the migraine attack, and a measure, a behavioral or physiological endpoint quantifying the response. Migraine-associated activation of nociceptive pathways leads to a lowered threshold for both mechanical and thermal stimuli, manifesting as hyperalgesia (exaggerated pain to harmful stimuli) and allodynia (pain in response to normally innocuous stimuli). These behaviors closely resemble clinical symptoms, providing high face validity to the models. Mechanical sensitivity is typically assessed using von Frey filaments [46], most commonly with the up–down

method (modified Dixon approach), which estimates the threshold eliciting a response in 50% of the animals based on a statistical formula [47].

Direct stimulation of the trigeminal neurons

Most animal models of migraine are based on the direct stimulation and activation of the trigeminal neurons *in vivo*. Common methods to induce the activation of the trigeminovascular system in animals are: i) electrical stimulation of the trigeminal ganglion; ii) electrical stimulation of nerve endings that innervate the meninges, and iii) chemical stimulation of nerve endings that innervate the meninges by local application of inflammatory substances [46].

The first two are robust models, and highly predictive of translational efficacy, but they are invasive and require anesthesia throughout the experiment. The chemical stimulation model, in contrast, allows experiments to be performed in awake animals, enabling behavioral assessments such as mechanical allodynia testing with von Frey filaments [46].

Exogenous administration of compounds

A hallmark of migraine is that a variety of external or internal triggers can provoke attacks. This concept is exploited in preclinical models through the systemic administration of substances that evoke migraine-like symptoms [48], [49]. Among these, nitric oxide (NO) donors, particularly glyceryl trinitrate (GTN) administered intraperitoneally, are the most commonly used. GTN's translational relevance lies in its ability to provoke migraine attacks in humans [50]. CGRP itself administered systemically can reproduce several migraine-associated symptoms such as photophobia, periorbital allodynia, and spontaneous pain following intraperitoneal or subcutaneous injection [51]–[53]. Additional migraine-inducing agents include adrenomedullin, amylin, PACAP, VIP, histamine, and prostaglandin E2, all of which elicit periorbital mechanical allodynia (PMA) in rodents [52].

Stress-induced migraine

In models employing an active dose of GTN 10 mg/kg, chronic stress paradigms, including restraint stress [54], chronic unpredictable stress, and social defeat stress [55], did not alter the pro-allodynic effects of GTN. Similarly, chronic variable stress failed to modify CSD in a genetic mouse model of migraine carrying the familial hemiplegic migraine type 1 (FHM1) mutation [12]. Conversely, Raoof et al. demonstrated that chronic unpredictable stress or maternal separation exacerbates

the effects of a lower, submaximal dose of GTN (5 mg/kg), an effect that was restricted to female animals [56]. These findings suggest that, in paradigms employing a fully effective trigger, a ceiling effect may occur, concealing any potential exacerbation of migraine-like pain due to prior stress exposure.

In contrast, when chronic stress precedes a subthreshold migraine inducer, a clear facilitatory effect of stress has been consistently reported. Avona et al. demonstrated that restraint stress (2 h per day for three consecutive days) induces transient periorbital mechanical allodynia, which resolves within 14 days after stress termination. Notably, once behavioral responses return to baseline, a subthreshold dose of the nitric oxide donor sodium nitroprusside (0.1 mg/kg) becomes effective in eliciting allodynia in previously stressed but not in naïve mice [57]. This condition, defined as “latent sensitization,” reflects a long-lasting increase in susceptibility to migraine triggers induced by prior stress exposure. In this model, sumatriptan was ineffective in reversing the effects of sodium nitroprusside, whereas a CGRP-targeting antibody fully abolished migraine-like symptoms [57]. In a separate study, pretreatment with the β-adrenergic blocker propranolol or the CGRP receptor antagonist olcegepant effectively prevented both allodynia and stress-induced priming, while sumatriptan successfully reversed umbellulone-induced symptoms [58]. These findings mirror the clinical utility of these compounds, with β-blockers and CGRP-pathway drugs approved for migraine prophylaxis and triptans indicated for acute attack management.

Interestingly, pretreatment with the selective kappa opioid receptor antagonist norbinaltorphimine (nor-BNI) prior to stress exposure prevented the development of stress-induced migraine priming, highlighting the kappa opioid receptor system as a promising pharmacological target for the prevention of stress-triggered migraine episodes [58], highlighting the potential of opioid research in this field.

1.2 THE NOCICEPTIN/ORPHANIN FQ – NOP RECEPTOR SYSTEM

In 1994, immediately after the cloning of mu, delta and kappa opioid receptors, a fourth G protein-coupled receptor (GPCR) with high sequence homology was discovered and named opioid receptor like 1 (ORL1) by Mollerau and colleagues. Despite structural similarities ORL1 could not be activated by standard opioid ligands. Its coupling proved to be via G_i protein, likely other opioid receptors, as adenylyl cyclase is inhibited upon its activation [59].

As a result of the first documented instance of reverse pharmacology (the process of identification of endogenous ligands subsequent to the cloning of their receptors) [60], one year later, the endogenous ligand, a 17 aa neuropeptide, was identified from rat and porcine brain extracts [61], [62]. Due to its ability to decrease hot plate latency when administered intracerebroventricularly (i.c.v.) in mice, this peptide was named Nociceptin. It is also referred to as Orphanin FQ, as it is the ligand of an orphan receptor and its sequence begins with phenylalanine (Phe) and ends with glutamine (Gln) [61]. This ligand – receptor system is now officially referred as Nociceptin Opioid Receptor (or NOP) and Nociceptin/Orphanin FQ (N/OFQ) [63].

The N/OFQ – NOP receptor system is widely expressed in the brain, spinal cord and peripheral tissues, where it has an important role in the modulation of various biological functions ranging from pain, mood, sleep, food intake, learning and memory, locomotion, to intestinal motility, cardiovascular homeostasis and immune response [64]. Consequently, the NOP receptor has received increasing attention as an innovative therapeutic target for different disorders.

1.3.1 NOP receptor

The NOP receptor is a GPCR characterized by seven transmembrane helices (TM) connected with intra (ICL)- and extracellular loops (ECL). Its primary structure is highly conserved among mammals, with human and murine sequences sharing more than 95% sequence identity . The NOP receptor is classified as a Class A (rhodopsin-like) GPCR, together with the classical opioid receptors, with which it shares conserved sequences in the intracellular loops and transmembrane domains [65]. Moreover, the coding and non-coding regions of the NOP gene (Oprl1) are similarly organized to those of the mu, delta and kappa receptors genes, suggesting evolution from a common ancestor gene [66]. TMs and ICL display very high conservation among opioid receptors, especially in regions mediating G proteins

interaction. On the other hand, extracellular loops, involved in ligand recognition and receptor activation, are highly divergent across receptors subtypes.

The crystal structure of the inactive NOP receptor was determined in complex with the peptide mimetic antagonist Compound-24 [67]. This compound mimics the first N-terminal residues of the NOP-selective antagonist UFP-101, a N/OFQ derivative, and binds deeply into the orthosteric pocket. Site directed mutagenesis studies identified the key residues responsible for NOP selectivity compared with classical morphinan opioid ligands [68]. Computational homology modeling has been instrumental in exploring the active-state conformation of NOP, paving the way for structure-based drug discovery (SBDD) [69]. Recently, deep-learning approaches have been used to generate in silico model of the activated NOP (NOPa) in complex with N/OFQ in its bioactive conformation [70].

NOP receptor distribution

The first comprehensive mapping of NOP receptor expression was performed in the adult rat CNS with (125)I-[(14)Tyr]-N/OFQ as the radioligand and *in situ* hybridization for its mRNA. The wide distribution of NOP throughout cortical and subcortical regions, i.e. hypothalamic nuclei, amygdala, and hippocampus, suggests the involvement of the N/OFQ – NOP system in numerous CNS functions, including nociception, reinforcement, stress and emotional regulation, motor control, and autonomic homeostasis [71]. NOP receptors are highly expressed along both ascending and descending pain pathways, including periaqueductal grey (PAG), thalamic nuclei, somatosensory cortex, dorsal medulla, spinal cord and dorsal root ganglia (DRG) [72]. Within these circuits, NOP often co-localizes with mu-opioid receptors, and its activation can either inhibit or modulate opioid-induced signaling depending on the neuronal context and anatomical region. For instance, descending pain pathways co-express NOP and mu receptors which cooperate in the inhibition of pain transmission. Although early studies described NOP activation as anti-opioid, current evidence indicates that it exerts complex, region-specific modulatory effects on opioid-mediated analgesia. Beyond nociception, NOP is also expressed in brain regions associated with reward, stress, and affective disorders, including the ventral tegmental area, nucleus accumbens, prefrontal cortex, and central amygdala [73]. The development of the NOP- enhanced green fluorescent protein (eGFP) knock-in mouse model provided a higher-resolution mapping compared to autoradiographic techniques [74]. Using this model, NOP-eGFP expression was

observed in CGRP positive afferent nociceptors in DRG and in protein kinase C gamma (PKCγ) positive interneurons of the dorsal horn, further suggesting a role in the modulation of pain transmission. A few years later, the same mouse model was used to investigate NOP plasticity in the DRG in a spinal nerve ligation (SNL) paradigm, demonstrating a downregulation of NOP-eGFP receptor in spinal laminae I and II of the dorsal horn and in DRG neurons of neuropathic mice [75]. These findings imply an adaptive reorganization of NOP signaling in chronic pain conditions.

NOP receptor signaling

NOP activation leads to a downstream signaling typical of GPCRs, beginning with the dissociation of the heterotrimeric G protein into Gα and Gβγ subunits. The Gα subunits coupled to opioid receptors belong primarily to the $G\alpha_{i/o}$ family, whose activation leads to a decrease in intracellular cAMP levels. In particular, a recent study conducted on rat and human post mortem tissues identified $G\alpha_{i-3}$ as the predominant G protein subtype coupled to NOP [76]. Similarly to other opioid receptors, NOP signaling via the Gβγ complex modulates the G protein–activated inwardly rectifying potassium channels (Kir3/GIRK) and voltage gated calcium channels [77]. Opening of Kir3 channel causes neuronal hyperpolarization and inhibition of excitability, whereas Gβγ-mediated inhibition of Ca^{2+} conductance further suppresses neurotransmitter release [78]. NOP activation also engages intracellular kinase cascades including PKA and protein kinase C (PKC) pathways [79], [80], along with mitogen-activated protein kinases (MAPK) pathways. In addition, c-Jun N-terminal kinase (JNK) can be activated through G-protein-dependent and independent mechanisms, involving G-protein Receptor Kinase (GRK)/ β-arrestin complexes [78].

NOP receptors, like mu, delta and kappa opioid receptors, undergo homologous desensitization, a process in which prolonged agonist exposure reduces receptor responsiveness. This multistep mechanism involves receptor phosphorylation, internalization, downregulation, and recycling. Following receptor activation, the Gβγ complex guides the recruitment of GRKs that phosphorylate the receptor C-terminal domain. This phosphorylation promotes conformational change, exposing docking sites for β-arrestin, facilitating receptor endocytosis. GRK2 and GRK3 are the principle serine/threonine kinase phosphorylating the NOP receptor [81],

initiating internalization though clathrin-dependent and independent pathways [82]. The predominant arrestin involved is arrestin 3 (β-arrestin2) which binds to a site requiring phosphorylation of Ser363. Mutation of this residue prevents NOP internalization [78].

1.3.2 Nociceptin/orphanin FQ

A few months after the discovery of NOP receptor, then still orphan of its endogenous ligand, two independent groups identified a neuropeptide with the same primary sequence, FGGFTGARKSARKLANQ, which they named N/OFQ [61], [62]. N/OFQ is generated from a larger precursor protein, prepronociceptin (ppN/OFQ), encoded by the PNOC gene, which undergoes post-translational processing by prohormone convertases to release the mature peptide. N/OFQ share high sequence similarity with dynorphin A, the endogenous ligand for the kappa receptor [61], [62]. However, due to its unique sequence, N/OFQ does not detectably interact with the classical opioid receptors (mu, delta, and kappa), and conversely, opioid peptides do not bind the NOP receptor. The first four N-terminal residues of N/OFQ form the “message domain”, responsible for receptor activation, while residues 7–17 constitute the “address domain”, mediating NOP receptor recognition [83]. The central dipeptide Thr^{5}-Gly^{6} acts as a hinge region connecting the two regions. The first four residues of N/OFQ resemble the canonical Tyr-Gly-Gly-Phe (YGGF) N-terminal motif of opioid peptides; however, substitution of Tyr^{1} with Phe^{1} confers NOP selectivity, preventing hydrogen bonding with the classical opioid receptor pocket.

Crystallographic studies indicate that the phenyl ring of Phe^{1} in N/OFQ is oriented toward a hydrophobic pocket of the receptor, whereas the phenolic group of Tyr^{1} in opioid peptides forms a hydrogen bond network with the conserved His^{52} in TM6 of opioid receptors [84].

Once released by neurons, N/OFQ is rapidly inactivated by proteolytic enzymes into biologically inactive fragments. In mouse brain N/OFQ inactivation is primarily mediated by aminopeptidase N (APN), which generates the inactive fragment N/OFQ(2–17). Endopeptidase 24.15 (EP 24.15) further cleaves the Ala^{7}–Arg^{8}, Ala^{11}–Arg^{12}, and Arg^{12}–Lys^{13} peptide bonds to produce inactive metabolite [85]. On the other hand, EP 24.11 (enkephalinase) does not appear to participate in N/OFQ degradation but can cleave the Lys^{13}–Leu^{14} bond, playing an early role in

neuropeptide metabolism [86]. Co-administration of EP 24.15 and APN inhibitors enhances the behavioral effects of N/OFQ in mice, confirming the role of these peptidases in its enzymatic inactivation [87].

1.3.3 Biological activities of Nociceptin/Orphanin FQ – NOP receptor system

The peptide N/OFQ participates in a wide range of physiological processes, with effects observed in the central and peripheral nervous system, in the cardiovascular system, in the airways, in the gastrointestinal tract, in the urogenital tract, and the immune system. It is well established that, at the spinal level, N/OFQ exerts an antinociceptive effect with many features common to other members of the opioid family. However, when administered supraspinally, N/OFQ may counteract the effects of opioids (anti-opioid action), producing hyperalgesia in animal models. In the brain, N/OFQ also modulates stress, anxiety, mood, reward, and locomotion [64].

Pain

Since its discovery, the N/OFQ–NOP receptor system has been extensively investigated for its role in pain modulation, revealing a complex and context-dependent profile. In rodents, NOP agonists exhibit effects ranging from pro-nociceptive to analgesic, depending on the route of administration (supraspinal, spinal, or peripheral) and on the pain model employed [88]–[90]. Supraspinal administration of N/OFQ or selective NOP agonists typically induce hyperalgesia in acute pain paradigms [61], [64], [91], [92], likely due to inhibition of OFF cells in the rostral ventromedial medulla (RVM), a key center in descending pain modulation [65], [89].

In contrast, intrathecal administration produces robust analgesia in both acute [93]–[95] and chronic pain models [96]–[98]. At the spinal level, NOP receptor activation inhibits nociceptive transmission by inhibiting glutamate release (via presynaptic calcium channel blockade) and by hyperpolarizing second-order neurons (via potassium channel opening) [65], [89]. Spinal N/OFQ appears to be more effective in chronic than in acute pain, although the underlying mechanisms remain under investigation.

Systemically administered non-peptide NOP agonists show limited efficacy in acute nociception [99], [100] but display robust antinociceptive activity in inflammatory pain models, such as the formalin test, carrageenan-induced hyperalgesia, and complete Freund's adjuvant (CFA)–induced inflammation. In these paradigms, compounds such as Ro 65-6570 and Ro 64-6198 significantly reduced licking behavior and thermal hyperalgesia, effects prevented by selective NOP antagonists, thus confirming receptor specificity [101], [102].

There are marked species differences in the efficacy and tolerability of systemically delivered NOP receptor agonists. In rodents systemic administration of the selective NOP agonist Ro 64-6198 fails to produce significant antinociceptive effects [100], [103], while in non-human primates it exhibits strong and effective thermal antinociception, without accompanying motor or sedative side effects [104]. In non-human primates, NOP agonists antinociceptive effects are naloxone-insensitive and free from classical opioid-related side effects such as itch, respiratory depression, and abuse potential [105]. Genetic deletion of N/OFQ or NOP in rodents enhanced inflammatory hyperalgesia in the formalin test, but not acute nociception [106], [107], further highlighting the adaptive, context-dependent nature of this system in pain processing.

Anxiety and depression

The N/OFQ – NOP receptor system is expressed in mood-regulating brain regions such as amygdala, hippocampus, thalamus and cortex [71], [108] . A large body of evidence indicates that NOP receptor activation exerts anxiolytic-like effects., I.c.v. administration of N/OFQ produces anxiolytic effects in rodents [109]. Likewise, NOP receptor agonists, including Ro 65-6570, Ro 64-6198 and SCH-221510 produced anxiolytic effects comparable to those of benzodiazepines, without inducing sedation or motor impairment [110]–[112].

Conversely, mice lacking the ppN/OFQ gene display an anxiogenic phenotype, indicating that endogenous N/OFQ signaling physiologically restrains anxiety responses [113]. This evidence, however, is only partially supported by findings in NOP knockout mice, which exhibit increased anxiety in some behavioral paradigms but not in others, and by studies showing that pharmacological NOP receptor antagonists do not alter baseline anxiety levels [114]–[116][117]. NOP receptor blockade or genetic deletion produces antidepressant-like effects in several preclinical models. NOP(-/-) animals or animals treated with selective antagonists

such as UFP-101 or SB-612111 exhibit reduced immobility in the forced swim and tail suspension tests, consistent with antidepressant behavior [115], [118]. Similar antidepressant effects have also been observed in other validated models of depression, including learned helplessness and chronic unpredictable mild stress paradigms, particularly after repeated administration of NOP antagonists, further supporting the robustness of these findings [119], [120]. The non-peptidic NOP receptor antagonist LY2940094 represented the first compound of this class to reach clinical evaluation in patients with major depressive disorder. Although its development was discontinued due to a lack of clear superiority over standard antidepressants, this compound provided the first clinical evidence that NOP receptor blockade is well tolerated and produces measurable antidepressant effects in humans [121].

Stress

Experimental stress models have provided compelling evidence that the N/OFQ–NOP receptor system plays an important modulatory role in stress-related behaviors. In rodents, pharmacological activation of NOP receptors prior to stress exposure consistently worsens stress outcomes, whereas NOP receptor blockade or genetic deletion exerts protective effects.

Specifically, pretreatment with NOP agonists has been shown to exacerbate the behavioral consequences of acute and chronic stress, including anxiety-like, depressive-like, and cognitive impairments [122], [123]. Conversely, administration of selective NOP antagonists or genetic NOP knockout prevents or mitigates these alterations, promoting resilience to stress and preserving normal emotional and cognitive performance [124]–[126]. These findings demonstrate that endogenous N/OFQ signaling contributes to the maladaptive response to stress, and that NOP inhibition may counteract stress-induced dysregulation of mood and cognition, likely through modulation of monoaminergic transmission and hypothalamic–pituitary–adrenal (HPA) axis activity [122], [124].

Exposure to stress can bidirectionally modulate pain perception, inducing analgesia (stress-induced analgesia, SIA) in acute settings or hyperalgesia (stress-induced hyperalgesia, SIH) when stress becomes chronic [127]. Activation of the N/OFQ–NOP receptor system during stress appears to facilitate SIH and impair SIA, likely by counteracting monoaminergic transmission and modulating the activity of the hypothalamic–pituitary–adrenal (HPA) axis [127].

Learning and memory

N/OFQ exerts adverse effects on learning processes and cognitive functions. Intra-17329551 hippocampal N/OFQ impairs spatial memory in rats [128], whereas NOP(-/-) mice displayed better learning abilities and long lasting memory than NOP(+/+) mice [129]. The detrimental effects of a single dose of N/OFQ or NOP receptor agonists on learning and memory have been consistently demonstrated across various tasks that heavily rely on hippocampal and amygdala function [130]. N/OFQ reduces neuronal excitability, inhibits long-term potentiation, and decreases the release of neurotransmitters crucial for synaptic plasticity and memory formation. Functional antagonism between NOP and NMDA receptors suggests that under pathological conditions, elevated N/OFQ could exacerbate cognitive deficits associated with impaired glutamatergic signaling. Notably, the NOP antagonists SB-612111 was able to prevent memory impairment elicited by chronic stress. Similarly, the genetic knock-down of NOP receptors prevented memory deficit in this model [131].

Sleep

NOP activation reduces locomotor activity and induces sedation-like effects in rodents [132]. Systemic Ro 64-6198 induced loss of righting reflex (LRR) in wild type (WT) but not in NOP(-/-) animals [133]. I.c.v. N/OFQ (10 nmol) induced hypoactivity without hypothermia [62], [134]. Ro 65-6570 and Org 26383 induced LRR and a suppression of burst activity in EEG recording in mice and rats [135]. A dose-dependent suppression of rapid eye movement (REM) sleep, an increase in non-REM sleep and slow wave activity were detected after the administration of Ro 64-6198 and SR16835 in rats, mice, and Cynomolgus macaques [132]. The NOP partial agonist sunobinop is the first NOP targeting drug tested in clinical trials for sleep disorders. 10 mg oral dose of sunobinop proved a positive effect on sleep/wake function in subjects with insomnia [136].

1.2.4 N/OFQ-NOP receptor system and migraine

Over the last two decades, growing experimental and clinical evidence has suggested that the N/OFQ system may represent an innovative target for the development of new antimigraine drugs. These findings are based on a wide range

of studies, including receptor localization analyses, *in vitro* experiments on primary trigeminal cells*, in vivo* investigations in anesthetized and freely moving animals, and clinical observations in migraine patients. Localization studies have shown that N/OFQ and NOP are expressed within key trigeminal structures critically involved in migraine pathophysiology. NOP mRNA and N/OFQ peptide have been identified in the trigeminal ganglion and in the spinal trigeminal nucleus of rats and mice using immunohistochemistry and transgenic NOP-eGFP reporter lines [71], [137], [138]. The presence of NOP has also been demonstrated in human trigeminal ganglia [139], [140], supporting the translational relevance of these findings.

I*n vitro* evidence further indicates that NOP activation modulates trigeminal excitability. In primary cultures of mouse trigeminal ganglion neurons, N/OFQ inhibited voltage-dependent calcium channel currents, producing an overall inhibitory effect on neuronal activity [141]. Consistently, NOP agonists reduced the release of CGRP induced by different stimuli, including capsaicin, veratridine, and bradykinin. These effects are prevented by the selective NOP antagonist [Nphe^1]N/OFQ(1–13)NH_2, confirming receptor specificity [142]. Collectively, these studies highlight the ability of NOP signaling to inhibit neuropeptide release and dampen trigeminal neuron excitability, two critical steps in migraine pathogenesis.

In vivo findings from anesthetized rats demonstrate that local application of N/OFQ within the trigeminal nucleus caudalis inhibits excitatory responses evoked by NMDA, AMPA, or noxious stimulation[142]–[145]. This inhibitory action may underlie the antinociceptive effects of N/OFQ at the spinal and trigeminal levels. Bartsch and colleagues showed that N/OFQ inhibited neurogenic vasodilation of the middle meningeal artery induced by electrical stimulation. This effect was abolished by the NOP antagonist [Nphe^1]N/OFQ(1–13)NH_2 [146]. Moreover, in behavioral models, the selective NOP agonist Ro 64-6198 alleviated GTN-induced allodynia and social withdrawal [138], [147].

Clinical observations also point in the same direction: plasma levels of N/OFQ are reduced in migraine patients, particularly during the first three hours of the attack [148], suggesting an imbalance of this modulatory system during migraine episodes.

Taken together, evidence from cellular, animal, and human studies converges to indicate that the N/OFQ–NOP system plays an inhibitory and protective role within the trigeminovascular network.

This consolidated body of data supports the view of NOP as a promising target for the development of innovative anti-migraine therapeutics. However, most available data derive from acute, pharmacologically induced models, and further research is required to assess the efficacy of NOP agonists in chronic or spontaneous forms of migraine, that more closely replicate the human experience.

AIM OF THE STUDY

The overall aim of this PhD project is to investigate the role of NOP receptor in migraine pathophysiology and to assess its potential as a novel therapeutic target for the treatment of this disorder.
In particular three specific aims (SA) have been developed:

SA1) *NOP validation in acute migraine model -* To define the specific contribution of the NOP receptor and its endogenous ligand, N/OFQ, in the acute migraine-like phenotype induced by CGRP, through the use of genetic deletion models; to determine if the peripheral activation of NOP receptors is sufficient to inhibit CGRP-evoked allodynia, through the use of both brain penetrant and peripherally restricted NOP agonists; to explore NOP endosomal signaling and its capacity to modulate CGRP-induced cAMP responses in human Schwann cells.

SA2) *NOP validation in a stress-induced migraine model* - to test whether NOP receptor activation prevents or reverses allodynia in a clinically relevant model of stress-induced migraine, through the use of both brain penetrant and peripherally restricted NOP agonists.

SA3) *Design, synthesis, and pharmacological characterization of novel NOP receptor agonists as new antimigraine drugs -* to develop novel, long-acting NOP receptor agonists by leveraging the N/OFQ dimerization strategy, with the primary objective of improving the pharmacological profile and therapeutic duration compared to the native peptide.

Collectively, these aims establish a comprehensive research pipeline directly aimed at the development of new anti-migraine drugs targeting the NOP receptor. This work integrates genetic, behavioral, pharmacological, and molecular approaches, combined with medicinal chemistry, to provide mechanistic and translational evidence necessary for proposing the NOP receptor as a robust and pharmacologically tractable target.

2 RESEARCH WORK

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

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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.

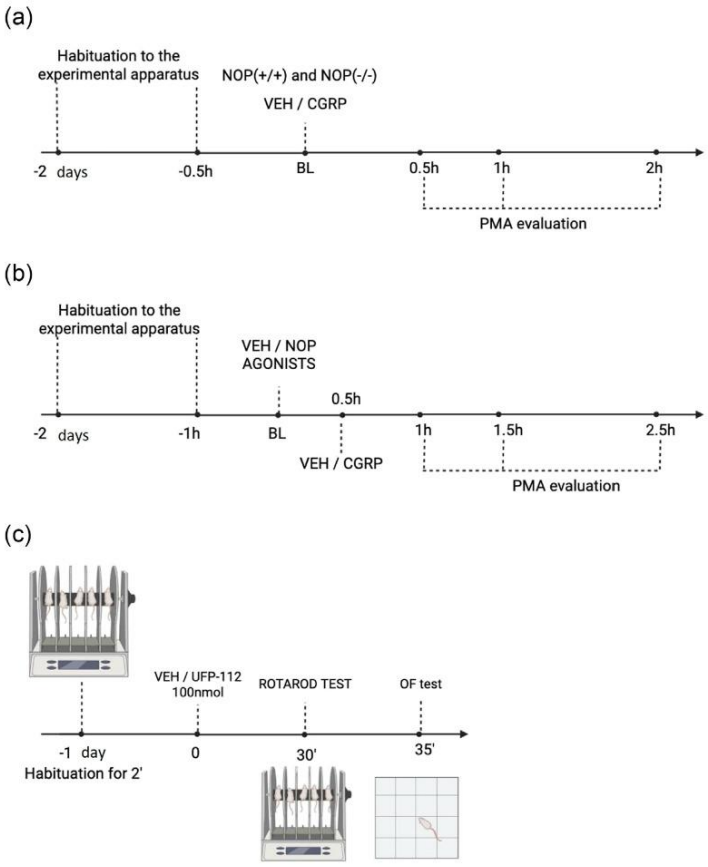

FIGURE 1 **Schematic representation of the in vivo experimental protocols used in this study.** (a) Schematic representation of the experimental timeline used to evaluate CGRP-induced Periorbital Mechanical Allodynia (PMA) across NOP(+/+) and NOP(−/−) mice. (b) Experimental design for assessing the effects 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$_{95\%}$.

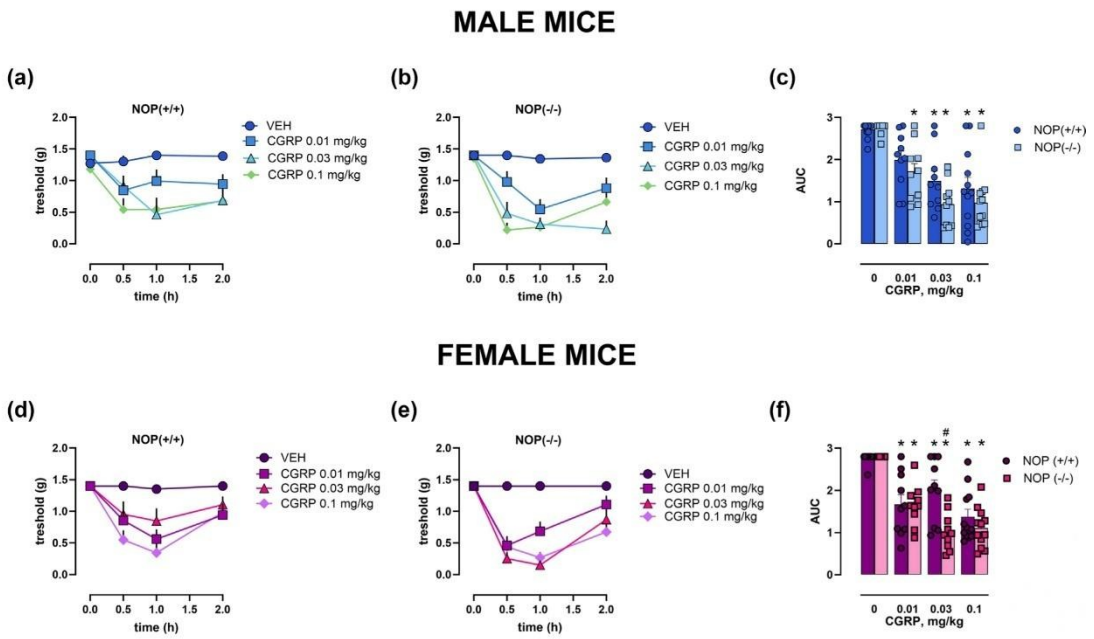

FIGURE 2 Effect of the i.p. administration of CGRP (0.01, 0.03 and 0.1 mg kg^{-1}) in NOP(+/+) and NOP(−/−) (a–c) male and (d–f) female mice. Threshold (g) was assessed 30 min, 1 h and 2 h following CGRP or vehicle administration. Figure 2a–e show the time course of the effect of CGRP, whereas Figure 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

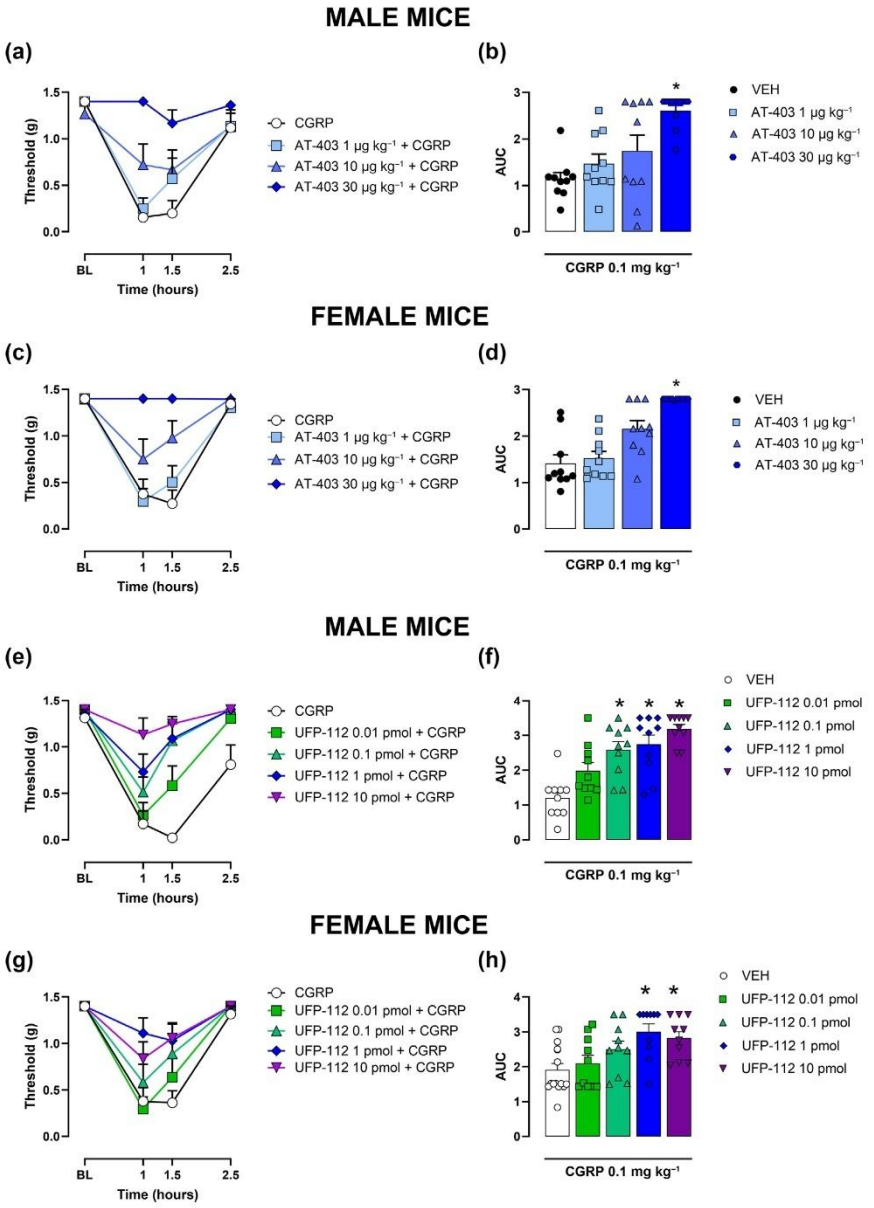

FIGURE 3 Effects of AT-403 and UFP-112 given i.p. 30 min before CGRP 0.1 mg kg^{-1} in CD-1 (a, b, e and f) male and (c, d, g and h) female mice. Sensitivity threshold (g) was assessed 1, 1.5 and 2 h following the administration of either vehicle, AT-403 or UFP-112. Left panels show the time course of 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.

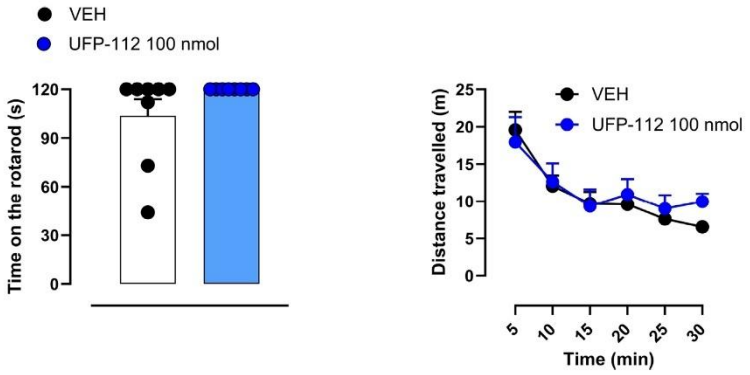

FIGURE 4 Effects of UFP-112 given i.p. in mice subjected to the (left) rotarod and (right) open field assays. Data are expressed as mean ± SEM, $n = 8$ mice per group. Total mice used for this experiment: 16. veh, vehicle.

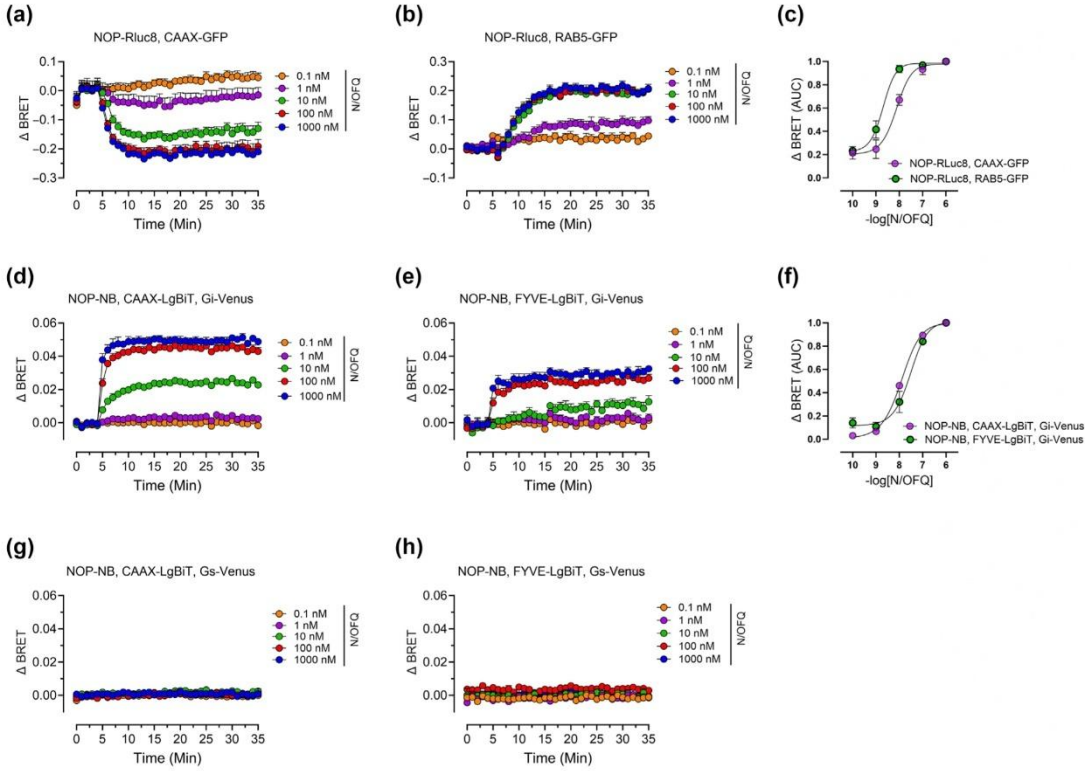

FIGURE 5 **BRET and nbBRET experiments in NOP-transfected HEK293 cells.** Effects of N/OFQ on NOP-CAAX (plasma membrane marker) and on NOP-RAB5 (endosome marker) interaction. (a and b) The time course of the effect, after subtraction of values from vehicle-treated wells. (c) The concentration–response 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.

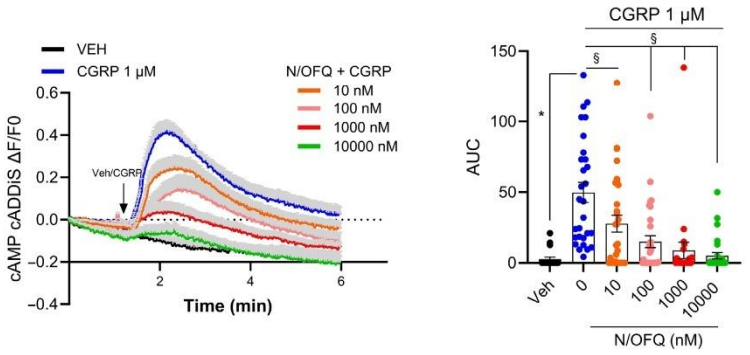

FIGURE 6 **Effects of CGRP and N/OFQ + CGRP on cAMP levels in human Schwann Cells (hSCs)**. Representative traces (the coloured line is the average trace of each experiment, in grey the SEM) and cumulative data (AUC) of cAMP formation induced by CGRP in the presence of different concentration of N/OFQ. 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 miniG$_{\alpha i}$ 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-miniG$_{\alpha s}$ 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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REFERENCES

Alexander, S. P. H., Christopoulos, A., Davenport, A. P., Kelly, E., Mathie, A. A., Peters, J. A., Veale, E. L., Armstrong, J. F., Faccenda, E., Harding, S. D., Davies, J. A., Abbracchio, M. P., Abraham, G., Agoulnik, A., Alexander, W., Al-hosaini, K., Bäck, M., Baker, J. G., Barnes, N. M., … Ye, R. D. (2023). The concise guide to pharmacology 2023/24: G protein-coupled receptors. *British Journal of Pharmacology*, *180*(Suppl 1), S23–S144.

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. *Bioorganic & Medicinal Chemistry*, *15*, 4434–4443. https://doi.org/10.1016/j.bmc.2007.04.026

Azevedo Neto, J., Ruzza, C., Sturaro, C., Malfacini, D., Pacifico, S., Zaveri, N. T., & Calò, G. (2021). Functional selectivity does not predict antinociceptive/locomotor impairing potencies of NOP receptor agonists. *Frontiers in Neuroscience*, *15*, 657153. https://doi.org/10.3389/fnins.2021.657153

Bartsch, T., Akerman, S., & Goadsby, P. J. (2002). The ORL-1 (NOP1) receptor ligand nociceptin/orphanin FQ (N/OFQ) inhibits neurogenic dural vasodilatation in the rat. *Neuropharmacology*, *43*, 991–998. https://doi.org/10.1016/S0028-3908(02)00148-X

Briscini, L., Corradini, L., Ongini, E., & Bertorelli, R. (2002). Up-regulation of ORL-1 receptors in spinal tissue of allodynic rats after sciatic nerve injury. *European Journal of Pharmacology*, *447*, 59–65. https://doi.org/10.1016/S0014-2999(02)01833-2

Calò, G., Rizzi, A., Cifani, C., Micioni Di Bonaventura, M. V., Regoli, D., Massi, M., Salvadori, S., Lambert, D. G., & Guerrini, R. (2011). UFP-112 a potent and long-lasting agonist selective for the nociceptin/orphanin FQ receptor. *CNS Neuroscience & Therapeutics*, *17*, 178–198. https://doi.org/10.1111/j.1755-5949.2009.00107.x

Capuano, A., Currò, D., Dello Russo, C., Tringali, G., Pozzoli, G., Di Trapani, G., Dello Russo, C., Di Trapani, G., & Navarra, P. (2007). Nociceptin (1-13)NH2 inhibits stimulated calcitonin-gene-related-peptide release from primary cultures of rat trigeminal ganglia neurones. *Cephalalgia*, *27*, 868–876. https://doi.org/10.1111/j.1468-2982.2007.01354.x

Capuano, A., De Corato, A., Tringali, G., Currò, D., Dello Russo, C., & Navarra, P. (2009). Buprenorphine inhibits bradykinin-induced release of calcitonin gene-related peptide from rat trigeminal neurons via both mu-opioid and nociceptin/orphanin peptide receptors. *European Journal of Pharmacology*, *609*, 45–50. https://doi.org/10.1016/j.ejphar.2009.03.013

Chaplan, S. R., Bach, F. W., Pogrel, J. W., Chung, J. M., & Yaksh, T. L. (1994). Quantitative assessment of tactile allodynia in the rat paw. *Journal of Neuroscience Methods*, *53*, 55–63. https://doi.org/10.1016/0165-0270(94)90144-9

Curtis, M. J., Alexander, S. P. H., Cortese-Krott, M., Kendall, D. A., Martemyanov, K. A., Mauro, C., Panettieri, R. A. Jr., Papapetropoulos, A., Patel, H. H., Santo, E. E., Schulz, R., Stefanska, B., Stephens, G. J., Teixeira, M. M., Vergnolle, N., Wang, X., & Ferdinandy, P. (2025). Guidance on the planning and reporting of experimental design and analysis. *British Journal of Pharmacology*, *182*(7), 1413–1415. https://doi.org/10.1111/bph.17441

De Logu, F., Landini, L., Janal, M. N., Li Puma, S., De Cesaris, F., Geppetti, P., & Nassini, R. (2019). Migraine-provoking substances evoke periorbital allodynia in mice. *The Journal of Headache and Pain*, *20*(1), 18. https://doi.org/10.1186/s10194-019-0968-1

De Logu, F., Nassini, R., Hegron, A., Landini, L., Jensen, D. D., Latorre, R., Ding, J., Marini, M., Souza Monteiro de Araujo, D., Ramírez-Garcia, P., & Whittaker, M. (2022). Schwann cell endosome CGRP signals elicit periorbital mechanical allodynia in mice. *Nature Communications*, *13*, 646. https://doi.org/10.1038/s41467-022-28204-z

Depner, U. B., Reinscheid, R. K., Takeshima, H., Brune, K., & Zeilhofer, H. U. (2003). Normal sensitivity to acute pain, but increased inflammatory hyperalgesia in mice lacking the nociceptin precursor polypeptide or the nociceptin receptor. *European Journal of Neuroscience*, *17*, 2381–2387. https://doi.org/10.1046/j.1460-9568.2003.02676.x

Dixon, W. J. (1980). Efficient analysis of experimental observations. *Annual Review of Pharmacology and Toxicology*, *20*, 441–462. https://doi.org/10.1146/annurev.pa.20.040180.002301

Edenson, S. (2020). Novel bioluminescent tools to study GPCR pharmacology in living cells. *The FASEB Journal*, *34*, 1–1. https://doi.org/10.1096/fasebj.2020.34.s1.02628

Edvinsson, J. C. A., Warfvinge, K., Krause, D. N., Blixt, F. W., Sheykhzade, M., Edvinsson, L., & Haanes, K. A. (2019). C-fibers may modulate adjacent Aδ-fibers through axon-axon CGRP signaling at nodes of Ranvier in the trigeminal system. *The Journal of Headache and Pain*, *20*(1), 105. https://doi.org/10.1186/s10194-019-1055-3

Edvinsson, L., Haanes, K. A., Warfvinge, K., & Krause, D. N. (2018). CGRP as the target of new migraine therapies - successful translation from bench to clinic. *Nature Reviews Neurology*, *14*, 338–350. https://doi.org/10.1038/s41582-018-0003-1

Ferrari, F., Malfacini, D., Journigan, B. V., Bird, M. F., Trapella, C., Guerrini, R., Lambert, D. G., Calo', G., & Zaveri, N. T. (2017). In vitro pharmacological characterization of a novel unbiased NOP receptor-selective nonpeptide agonist AT-403. *Pharmacology Research & Perspectives*, *5*(4), e00333. https://doi.org/10.1002/prp2.333

Fisher, N. M., & von Zastrow, M. (2024). Opioid receptors reveal a discrete cellular mechanism of endosomal G protein activation. *Proceedings of the National Academy of Sciences of the United States of America*, *122*(17), e2420623122. https://doi.org/10.1073/pnas.2420623122

Flores, C. A., Wang, X. M., Zhang, K. M., & Mokha, S. S. (2001). Orphanin FQ produces gender-specific modulation of trigeminal nociception: Behavioral and electrophysiological observations. *Neuroscience*, *105*, 489–498. https://doi.org/10.1016/S0306-4522(01)00179-8

GBD 2019 Diseases and injuries collaborators. (2020). Global burden of 369 diseases and injuries in 204 countries and territories, 1990-2019: A systematic analysis for the Global Burden of Disease Study 2019. *The Lancet*, *396*, 1204–1222. https://doi.org/10.1016/S0140-6736(20)30925-9

Giuliani, S., Tramontana, M., Lecci, A., & Maggi, C. A. (1997). Effect of nociceptin on heart rate and blood pressure in anaesthetized rats. *European Journal of Pharmacology*, *333*, 177–179.

Guerrini, R., Calo, G., Rizzi, A., Bianchi, C., Lazarus, L. H., Salvadori, S., Temussi, P. A., & Regoli, D. (1997). Address and message sequences for the nociceptin receptor: A structure-activity study of nociceptin-(1-13)-peptide amide. *Journal of Medicinal Chemistry*, *40*, 1789–1793. https://doi.org/10.1021/jm970011b

Hautakangas, H., Winsvold, B. S., Ruotsalainen, S. E., Bjornsdottir, G., Harder, A. V. E., Kogelman, L. J. A., Thomas, L. F., Noordam, R., Benner, C., Gormley, P., Artto, V., Banasik, K., Bjornsdottir, A., Boomsma, D. I., Brumpton, B. M., Burgdorf, K. S., Buring, J. E., Chalmer, M. A., de Boer, I., … Pirinen, M. (2022). Genome-wide analysis of 102,084 migraine cases identifies 123 risk loci and subtype-specific risk alleles. *Nature Genetics*, *54*(2), 152–160. https://doi.org/10.1038/s41588-021-00990-0

Hawes, B. E., Graziano, M. P., & Lambert, D. G. (2000). Cellular actions of nociceptin: Transduction mechanisms. *Peptides*, *21*, 961–967. https://doi.org/10.1016/S0196-9781(00)00232-1

Hegron, A., Peach, C. J., Tonello, R., Seemann, P., Teng, S., Latorre, R., Huebner, H., Weikert, D., Rientjes, J., Veldhuis, N. A., Poole, D. P., Jensen, D. D., Thomsen, A. R. B., Schmidt, B. L., Imlach, W. L., Gmeiner, P., & Bunnett, N. W. (2023). Therapeutic antagonism of the neurokinin 1 receptor in endosomes provides sustained pain relief. *Proceedings of the National Academy of Sciences of the United States of America*, *120*, e2220979120. https://doi.org/10.1073/pnas.2220979120

Holanda, V. A. D., Pacifico, S., Azevedo Neto, J., Finetti, L., Lobão-Soares, B., Calo, G., Gavioli, E. C., & Ruzza, C. (2019). Modulation of the NOP receptor signaling affects resilience to acute stress. *Journal of Psychopharmacology (Oxford)*, *33*, 1540–1549. https://doi.org/10.1177/0269881119864942

Hou, M., Uddman, R., Tajti, J., & Edvinsson, L. (2003). Nociceptin immunoreactivity and receptor mRNA in the human trigeminal ganglion. *Brain Research*, *964*, 179–186. https://doi.org/10.1016/S0006-8993(02)03927-6

Jimenez-Vargas, N. N., Gong, J., Wisdom, M. J., Jensen, D. D., Latorre, R., Hegron, A., Teng, S., DiCello, J. J., Rajasekhar, P., Veldhuis, N. A., Carbone, S. E., Yu, Y., Lopez-Lopez, C., Jaramillo-Polanco, J., Canals, M., Reed, D. E., Lomax, A. E., Schmidt, B. L., Leong, K. W., … Poole, D. P. (2020). Endosomal signaling of delta opioid receptors is an endogenous mechanism and therapeutic target for relief from inflammatory pain. *Proceedings of the National Academy of Sciences of the United States of America*, *117*, 15281–15292. https://doi.org/10.1073/pnas.2000500117

Johnson, K. W., Morin, S. M., Wroblewski, V. J., & Johnson, M. P. (2019). Peripheral and central nervous system distribution of the CGRP neutralizing antibody [(125)I] galcanezumab in male rats. *Cephalalgia*, *39*, 1241–1248. https://doi.org/10.1177/0333102419844711

Kiguchi, N., Ding, H., Kishioka, S., & Ko, M.-C. (2020). Nociceptin/orphanin FQ peptide receptor-related ligands as novel analgesics. *Current Topics in Medicinal Chemistry*, *20*, 2878–2888. https://doi.org/10.2174/1568026620666200508082615

Kunselman, J. M., Gupta, A., Gomes, I., Devi, L. A., & Puthenveedu, M. A. (2021). Compartment-specific opioid receptor signaling is selectively modulated by different dynorphin peptides. *eLife*, *10*, e60270. https://doi.org/10.7554/eLife.60270

Lambert, D. G. (2008). The nociceptin/orphanin FQ receptor: A target with broad therapeutic potential. *Nature Reviews Drug Discovery*, *7*, 694–710. https://doi.org/10.1038/nrd2572

Latorre, R., Hegron, A., Peach, C. J., Teng, S., Tonello, R., Retamal, J. S., Klein-Cloud, R., Bok, D., Jensen, D. D., Gottesman-Katz, L., Rientjes, J., Veldhuis, N. A., Poole, D. P., Schmidt, B. L., Pothoulakis, C. H., Rankin, C., Xie, Y., Koon, H. W., & Bunnett, N. W. (2022). Mice expressing fluorescent PAR2 reveal that endocytosis mediates colonic inflammation and pain. *Proceedings of the National Academy of Sciences of the United States of America*, *119*, e2112059119. https://doi.org/10.1073/pnas.2112059119

Lilley, E., Stanford, S. C., Kendall, D. E., Alexander, S. P. H., Cirino, G., Docherty, J. R., George, C. H., Insel, P. A., Izzo, A. A., Ji, Y., Panettieri, R. A., Sobey, C. G., Stefanska, B., Stephens, G., Teixeira, M., & Ahluwalia, A. (2020). ARRIVE 2.0 and the *British Journal of Pharmacology*: Updated guidance for 2020. *British Journal of Pharmacology*, *177*, 3611–3616. https://doi.org/10.1111/bph.15178

Lipton, R. B., Bigal, M. E., Ashina, S., Burstein, R., Silberstein, S., Reed, M. L., Serrano, D., Stewart, W. F., & American Migraine Prevalence Prevention Advisory Group. (2008). Cutaneous allodynia in the migraine population. *Annals of Neurology*, *63*, 148–158. https://doi.org/10.1002/ana.21211

Madeddu, P., Salis, M. B., Milia, A. F., Emanueli, C., Guerrini, R., Regoli, D., & Calò, G. (1999). Cardiovascular effects of nociceptin in unanesthetized mice. *Hypertension*, *33*, 914–919. https://doi.org/10.1161/01.HYP.33.3.914

Mason, B. N., Kaiser, E. A., Kuburas, A., Loomis, M. M., Latham, J. A., Garcia-Martinez, L. F., & Russo, A. F. (2017). Induction of migraine-like photophobic behavior in mice by both peripheral and central CGRP mechanisms. *Journal of Neuroscience*, *37*, 204–216. https://doi.org/10.1523/JNEUROSCI.2967-16.2016

McNamara, C. R., Mandel-Brehm, J., Bautista, D. M., Siemens, J., Deranian, K. L., Zhao, M., Hayward, N. J., Chong, J. A., Julius, D., Moran, M. M., & Fanger, C. M. (2007). TRPA1 mediates formalin-induced pain. *Proceedings of the National Academy of Sciences of the United States of America*, *104*, 13525–13530. https://doi.org/10.1073/pnas.0705924104

Melo-Carrillo, A., Strassman, A. M., Nir, R. R., Schain, A. J., Noseda, R., Stratton, J., & Burstein, R. (2017). Fremanezumab—A humanized monoclonal anti-CGRP antibody—Inhibits thinly myelinated (Aδ) but not unmyelinated (C) meningeal nociceptors. *The Journal of Neuroscience*, *37*(44), 10587–10596. https://doi.org/10.1523/JNEUROSCI.2211-17.2017

Meunier, J. C., Mollereau, C., Toll, L., Suaudeau, C., Moisand, C., Alvinerie, P., Butour, J. L., Guillemot, J. C., Ferrara, P., Monsarrat, B., Mazarguil, H., Vassart, G., Parmentier, M., & Costentin, J. (1995). Isolation and structure of the endogenous agonist of opioid receptor-like ORL1 receptor. *Nature*, *377*, 532–535. https://doi.org/10.1038/377532a0

Neal, C. R., Akil, H., & Watson, S. J. (2001). Expression of orphanin fq and the opioid receptor-like (ORL1) receptor in the developing human and rat brain. *Journal of Chemical Neuroanatomy*, *22*, 219–249. https://doi.org/10.1016/s0891-0618(01)00135-1

Neal, C. R., Mansour, A., Reinscheid, R., Nothacker, H. P., Civelli, O., & Watson, S. J. (1999). Localization of orphanin FQ (nociceptin) peptide and messenger RNA in the central nervous system of the rat. *Journal of Comparative Neurology*, *406*, 503–547.

Nishi, M., Houtani, T., Noda, Y., Mamiya, T., Sato, K., Doi, T., Kuno, J., Takeshima, H., Nukada, T., Nabeshima, T., & Yamashita, T. (1997). Unrestrained nociceptive response and disregulation of hearing ability in mice lacking the nociceptin/orphaninFQ receptor. *The EMBO Journal*, *16*, 1858–1864.

Noseda, R., Schain, A. J., Melo-Carrillo, A., Tien, J., Stratton, J., Mai, F., Strassman, A. M., & Burstein, R. (2020). Fluorescently-labeled fremanezumab is distributed to sensory and autonomic ganglia and the dura but not to the brain of rats with uncompromised blood brain barrier. *Cephalalgia*, *40*(3), 229–240. https://doi.org/10.1177/0333102419896760

Ozawa, A., Brunori, G., Mercatelli, D., Wu, J., Cippitelli, A., Zou, B., Xie, X. (S.), Williams, M., Zaveri, N. T., Low, S., Scherrer, G., Kieffer, B. L., & Toll, L. (2015). Knock-in mice with NOP-eGFP receptors identify receptor cellular and regional localization. *The Journal of Neuroscience*, *35*, 11682–11693. https://doi.org/10.1523/JNEUROSCI.5122-14.2015

Pavelic, A. R., Wöber, C., Riederer, F., & Zebenholzer, K. (2022). Monoclonal antibodies against calcitonin gene-related peptide for migraine prophylaxis: A systematic review of real-world data. *Cells*, *12*, 143. https://doi.org/10.3390/cells12010143

Peach, C. J., Kilpatrick, L. E., Woolard, J., & Hill, S. J. (2021). Use of NanoBiT and NanoBRET to monitor fluorescent VEGF-A binding kinetics to VEGFR2/NRP1 heteromeric complexes in living cells. *British Journal of Pharmacology*, *178*, 2393–2411. https://doi.org/10.1111/bph.15426

Pellesi, L., Do, T. P., & Hougaard, A. (2024). Pharmacological management of migraine: Current strategies and future directions. *Expert Opinion on Pharmacotherapy*, *25*, 673–683. https://doi.org/10.1080/14656566.2024.2349791

Percie du Sert, N., Hurst, V., Ahluwalia, A., Alam, S., Avey, M. T., Baker, M., Browne, W. J., Clark, A., Cuthill, I. C., Dirnagl, U., Emerson, M., Garner, P., Holgate, S. T., Howells, D. W., Karp, N. A., Lazic, S. E., Lidster, K., MacCallum, C. J., Macleod, M., … Würbel, H. (2020). The ARRIVE guidelines 2.0: Updated guidelines for reporting animal

research. *PLoS Biology*, *18*(7), e3000410. https://doi.org/10.1371/journal.pbio.3000410

Rea, B. J., Wattiez, A. S., Waite, J. S., Castonguay, W. C., Schmidt, C. M., Fairbanks, A. M., Robertson, B. R., Brown, C. J., Mason, B. N., Moldovan-Loomis, M. C., Garcia-Martinez, L. F., Poolman, P., Ledolter, J., Kardon, R. H., Sowers, L. P., & Russo, A. F. (2018). Peripherally administered calcitonin gene-related peptide induces spontaneous pain in mice: Implications for migraine. *Pain*, *159*, 2306–2317. https://doi.org/10.1097/j.pain.0000000000001337

Reinscheid, R. K., Nothacker, H. P., Bourson, 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. https://doi.org/10.1126/science.270.5237.792

Rizzi, A., Cerlesi, M. C., Ruzza, C., Malfacini, D., Ferrari, F., Bianco, S., Costa, T., Guerrini, R., Trapella, C., & Calò, G. (2016). Pharmacological characterization of cebranopadol, a novel analgesic acting as mixed nociceptin/orphanin FQ and opioid receptor agonist. *Pharmacology Research & Perspectives*, *4*(4), e00247. https://doi.org/10.1002/prp2.247

Rizzi, A., Molinari, S., Marti, M., Marzola, G., & Calò, G. (2011). Nociceptin/orphanin FQ receptor knockout rats: In vitro and in vivo studies. *Neuropharmacology*, *60*, 572–579. https://doi.org/10.1016/j.neuropharm.2010.12.010

Rizzi, A., Nazzaro, C., Marzola, G. G., Zucchini, S., Trapella, C., Guerrini, R., Zeilhofer, H. U., Regoli, D., & Calo', G. (2006). Endogenous nociceptin/orphanin FQ signalling produces opposite spinal antinociceptive and supraspinal pronociceptive effects in the mouse formalin test: Pharmacological and genetic evidences. *Pain*, *124*, 100–108. https://doi.org/10.1016/j.pain.2006.03.021

Rizzi, A., Ruzza, C., Bianco, S., Trapella, C., & Calò, G. (2017). Antinociceptive action of NOP and opioid receptor agonists in the mouse orofacial formalin test. *Peptides*, *94*, 71–77. https://doi.org/10.1016/j.peptides.2017.07.002

Rizzi, A., Spagnolo, B., Wainford, R. D., Fischetti, C., Guerrini, R., Marzola, G., Baldisserotto, A., Salvadori, S., Regoli, D., Kapusta, D. R., & Calo, G. (2007). In vitro and in vivo studies on UFP-112, a novel potent and long lasting agonist selective for the nociceptin/orphanin FQ receptor. *Peptides*, *28*, 1240–1251. https://doi.org/10.1016/j.peptides.2007.04.020

Romero-Reyes, M., & Akerman, S. (2014). Update on animal models of migraine. *Current Pain and Headache Reports*, *18*, 462. https://doi.org/10.1007/s11916-014-0462-z

Steiner, T. J., Stovner, L. J., Jensen, R., Uluduz, D., Katsarava, Z., & Lifting the burden: The global campaign against headache. (2020). Migraine remains second among the world's causes of disability, and first among young women: Findings from GBD2019. *The Journal of Headache and Pain*, *21*, 137. https://doi.org/10.1186/s10194-020-01208-0

Stewart, W. F., Wood, C., Reed, M. L., Roy, J., Lipton, R. B., & AMPP Advisory Group. (2008). Cumulative lifetime migraine incidence in women and men. *Cephalalgia*, *28*, 1170–1178. https://doi.org/10.1111/j.1468-2982.2008.01666.x

Sturaro, C., Fakhoury, B., Targowska-Duda, K. M., Zribi, G., Schoch, J., Ruzza, C., Calò, G., Toll, L., & Cippitelli, A. (2023). Preclinical effects of cannabidiol in an experimental model of migraine. *Pain*, *164*, 2540–2552. https://doi.org/10.1097/j.pain.0000000000002960

Targowska-Duda, K. M., Ozawa, A., Bertels, Z., Cippitelli, A., Marcus, J. L., Mielke-Maday, H. K., Zribi, G., Rainey, A. N., Kieffer, B. L., Pradhan, A. A., & Toll, L. (2020). NOP receptor agonist attenuates nitroglycerin-induced migraine-like symptoms in mice. *Neuropharmacology*, *170*, 108029. https://doi.org/10.1016/j.neuropharm.2020.108029

Titiz, M., Landini, L., Monteiro, S., de Araujo, D., Marini, M., Seravalli, V., Chieca, M., Pensieri, P., Montini, M., De Siena, G., Pasquini, B., Vannuccini, S., Iannone, L. F., Cunha, T. M., Brancolini, G., Bellantoni, E., Scuffi, I., Mastricci, A., Tesi, M., … De Logu, F. (2024). Schwann cell C5aR1 co-opts inflammasome NLRP1 to sustain pain in a mouse model of endometriosis. *Nature Communications*, *15*, 10142. https://doi.org/10.1038/s41467-024-54486-6

Wang, X. M., Zhang, K. M., & Mokha, S. S. (1996). Nociceptin (orphanin FQ), an endogenous ligand for the QRL1 (opioid-receptor-like1) receptor; modulates responses of trigeminal neurons evoked by excitatory amino acids and somatosensory stimuli. *Journal of Neurophysiology*, *76*, 3568–3572. https://doi.org/10.1152/jn.1996.76.5.3568

Whiteside, G. T., Kyle, D. J., Kapil, R. P., Cipriano, A., He, E., Zhou, M., Shet, M. S., Hummel, M., Knappenberger, T., Fukumura, K., Matsuo, Y., Uehira, M., Hiroyama, S., Takai, N., Willsie, S. K., & Harris, S. C. (2024). The nociceptin/orphanin FQ receptor partial agonist sunobinop promotes non-REM sleep in rodents and patients with insomnia. *Journal of Clinical Investigation*, *134*(1), e171172. https://doi.org/10.1172/JCI171172

Witta, J., Palkovits, M., Rosenberger, J., & Cox, B. M. (2004). Distribution of nociceptin/orphanin FQ in adult human brain. *Brain Res.*, *997*, 24–29. https://doi.org/10.1016/j.brainres.2003.08.066

Woodcock, A., McLeod, R. L., Sadeh, J., & Smith, J. A. (2010). The efficacy of a NOP1 agonist (SCH486757) in subacute cough. *Lung*, *188*(Suppl 1), S47–S52.

Xie, G. X., Meuser, T., Pietruck, C., Sharma, M., & Palmer, P. P. (1999). Presence of opioid receptor-like (ORL1) receptor mRNA splice variants in peripheral sensory and sympathetic neuronal ganglia. *Life Sciences*, *64*, 2029–2037. https://doi.org/10.1016/S0024-3205(99)00150-2

Zobdeh, F., Ben Kraiem, A., Attwood, M. M., Chubarev, V. N., Tarasov, V. V., Schiöth, H. B., & Mwinyi, J. (2021). Pharmacological treatment of migraine: Drug classes, mechanisms of action, clinical trials and new treatments. *British Journal of Pharmacology*, *178*, 4588–4607. https://doi.org/10.1111/bph.15657

SUPPORTING INFORMATION

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2.2 NOP receptor activation dampens stress-induced migraine-like behaviors in mice

NOP receptor activation counteracts stress-induced migraine-like behaviors in mice

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Keywords: nociceptin/orphanin FQ, NOP receptor, stress-induced periorbital mechanical allodynia, AT-403, UFP-112, migraine

Abstract

Stress is a major trigger of migraine, yet the underlying mechanisms remain unclear. We evaluated the role of the nociceptin/orphanin FQ (N/OFQ)/NOP receptor system in a mouse model where repeated restraint stress induces periorbital mechanical allodynia (PMA) and primes responses to a subthreshold dose of nitroglycerin (GTN, 0.1 mg/kg). Stress-induced PMA resolved in young mice but persisted for weeks in older animals, modelling chronic migraine. GTN, inactive in naïve mice, evoked PMA only in previously stressed animals. NOP knockout did not alter susceptibility, whereas pharmacological activation with the brain-penetrant NOP agonist AT-403 (30 µg/kg) or the peripherally restricted agonist UFP-112 (10 pmol) prevented GTN-evoked PMA and reversed stress-induced PMA, including persistent allodynia in aged mice. Comparable efficacy of UFP-112 and AT-403 highlights the contribution of peripheral NOP receptors. Together, our findings expand the evidence beyond acute pharmacological models, supporting the potential of NOP agonists for the treatment of both acute and chronic migraine.

1. INTRODUCTION

Migraine affects around 10–15% of the adult population worldwide and represents a major health burden [49,50]. Despite several available treatments, many patients remain unsatisfied, and even the recent advances with CGRP-targeting drugs leave a proportion of individuals insufficiently responsive, emphasizing the need for novel therapeutic options [40,41].

Nociceptin/orphanin FQ (N/OFQ) is the natural ligand of the NOP receptor, a G-protein coupled receptor (GPCR) discovered in the mid-1990s [34,44]. Structurally related to classical opioid receptors, NOP mediates inhibitory signaling by reducing cAMP formation, inhibiting voltage-gated Ca^{2+} currents, and activating inwardly rectifying K^{+} channels [21]. Evidence from rodent [24,29,36,39,53,58,60] and human [24,58] studies shows that NOP and N/OFQ are expressed in trigeminal structures relevant to migraine. Here, NOP activation reduces CGRP release and attenuates trigeminal excitability [8,9,16,54]. Importantly, N/OFQ administration was found to reduce neurogenic dural vasodilation [4], and the selective NOP agonist Ro 64-6198 alleviated allodynia and social deficits in a nitroglycerin (GTN)-induced mouse model of migraine [35,53]. More recently, our group demonstrated that peripheral NOP activation is sufficient to prevent CGRP-induced periorbital mechanical allodynia (PMA) [52]. Collectively, these data strengthen the rationale for considering NOP as a promising target for novel anti-migraine strategies. However, current studies have so far addressed only acute, pharmacologically induced migraine-like responses, and it remains to be clarified whether NOP agonists are also effective in chronic models or when migraine-like behaviours are triggered by stimuli more closely resembling the human condition.

Clinical observations consistently indicate that many patients identify stress as one of the most common precipitating factors for migraine attacks [28]. To investigate the mechanisms underlying this association, preclinical studies have used paradigms in which stress is applied before exposure

to established migraine triggers. In this context, Avona et al. showed that three consecutive days of 2-hour restraint stress induced PMA in mice, which spontaneously resolved within two weeks. Remarkably, once the animals had returned to baseline, a normally ineffective (subthreshold) dose of the nitric oxide donor sodium nitroprusside (0.1 mg/kg) was sufficient to provoke migraine-like responses, but only in previously stressed mice [2]. This phenomenon, referred to as "latent sensitization," suggests that stress can increase vulnerability to subsequent triggers. Similar effects have been reproduced with other chemical inducers, including the TRPA1 agonist umbellulone [31] and pituitary adenylate cyclase-activating polypeptide-38 (PACAP38) [48]. Clinical evidence shows that stress heightens vulnerability to non-noxious stimuli in migraineurs [13,43,51].

In this study, we aimed to evaluate the role of NOP receptor modulation in a mouse model of migraine, based on chronic restraint stress followed by the administration of a subthreshold dose of GTN. To investigate NOP blockade, we employed NOP wild-type (NOP(+/+)) and knockout (NOP(–/–)) mice. For NOP activation, two selective agonists were tested: the brain-penetrant AT-403 [15] and the peripherally restricted UFP-112 [46]. We assessed the ability of these approaches to prevent GTN-induced responses after stress priming, as well as to affect the direct effects of stress on PMA.

2. MATERIALS AND METHODS

2.1. Materials

GTN, from Bioindustria LIM S.p.A. (Novi Ligure, Italy), was used at a dose of 0.1 mg/kg and solubilized in 5% glucose and 1.5% propylene glycol in distilled water. AT-403 was provided by Dr. Nurulain Zaveri (Astraea Therapeutics, Mountain View, CA, USA) and dissolved in 1% dimethyl sulfoxide (DMSO), 0.3% Cyclodextrin, and distilled water. UFP-112 was synthesized in-house, as previously described [1,20], and dissolved in PBS. AT-403 (30 µg/kg), UFP-112 (10 pmol), and their vehicles were injected

intraperitoneally (i.p.) in a volume of 10 ml/kg. The doses of AT-403 and UFP-112 used in this study have been selected based on a previous dose-response curve study performed in our laboratory, demonstrating that at these doses, both compounds can fully prevent the PMA induced by CGRP [52].

2.2. Animals

All the experimental procedures adopted in the in vivo studies comply with the European Directive 2010/63/EU on protecting 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 (authorization number 692/2022-PR). In vivo studies have been reported following ARRIVE guidelines [30]. 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 a 12-hour light/dark cycle. Food and water were provided ad libitum. CD-1 male mice, aged 3 to 4 months or 9 months, were used. Details about the generation of NOP(-/-) and NOP(+/+) mice have been published previously [5,37]; these mice have been backcrossed on CD-1 strain in our laboratories. NOP(+/+) and NOP(-/-) littermates were obtained by mating NOP(+/-) mice. All mice were genotyped as described [23]. Each mouse was used only once.

2.3. Stress exposure

Stress induction was performed by placing mice in conical plastic restrainers that prevented movement while allowing free respiration through multiple holes at the bottom of the tube. On the first day, animals were introduced into the restrainers by gently guiding them forward, avoiding trauma from tail threading or improper insertion. The procedure was carried out for 2 hours per day over 4 consecutive days (Figure 1). Sham animals were housed in a separate room from stressed

animals for the entire duration of the stress protocol. During restraint, animals were monitored every 15 minutes to confirm that they maintained the correct position; if an animal shifted, it was readjusted by the experimenter. Restraint devices were adjusted according to body weight: mice ≥40 g were placed in disposable cardboard tubes with a ventilated metal grid, while mice <30 g were provided with filler paper to ensure proper restraint. To prevent transmission of stress-related phenotypes, stressed and sham animals were not co-housed. A mouse was considered susceptible to stress when its periorbital mechanical threshold was below 0.4 g in at least one of the measurements at 1, 7, or 14 DPS.

2.4. 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 minutes two days before the experiment and again for 30 minutes immediately before the experiment began. Periorbital mechanical allodynia (PMA) was measured using von Frey filaments (Ugo Basile srl, Varese, Italy) according to the up-and-down paradigm (Dixon, 1980; Chaplan et al., 1994). 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 slight buckling, and held for about 5 seconds 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:

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

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 [10]. The baseline measurement was assessed before the stress protocol. Then, PMA was measured 1, 7, 14, 21 days post stress (DPS) and 60' after GTN administration, if not stated otherwise (Figure 1). Experiments were performed between 9:00 a.m. and 2:00 p.m.

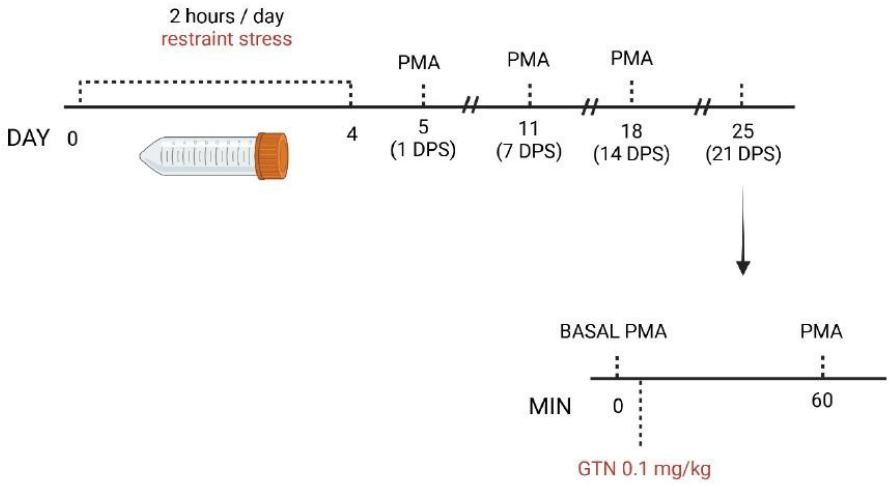

Figure 1. Experimental timeline. Schematic representation of the study design showing the sequence and duration of experimental procedures.

2.5. Statistical analysis

The experimental sample sizes were determined a priori by performing a power analysis using GPower 3.1 software. Power analysis was conducted based on the following criteria: effect size = 0.7, β =

0.8, and $\alpha = 0.05$. This resulted in the requirement of a minimum of 8 mice per group to achieve adequate statistical power. In stressed groups, group sizes were increased to account for variability in stress responsiveness, to always ensure $N \geq 8$ per group. The precise number of mice in each experimental group is detailed in the figure legends. A total of 221 animals were used in this study. Animals resilient to stress effects were excluded from the study (53 mice out of 200 stressed mice, 26%), as well as mice that still shown PMA at 21 DPS (2 mice out of 200 stressed mice). A mouse was considered susceptible when its periorbital mechanical threshold was below 0.4 g in at least one of the measurements at 1, 7, or 14 DPS. Mice were randomly assigned to pharmacological treatments. 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 analyzed using GraphPad Prism 10.5 software. Data were analyzed using one- or two-way analysis of variance (ANOVA). Multiple comparisons were made using Tukey's or Bonferroni's post hoc tests only if the ANOVA test yielded significant results. Differences were considered statistically significant at $p < 0.05$. The statistical tests used, and corresponding statistical values, are noted in the figure legends.

3. RESULTS

3.1. NOP receptor deletion does not affect the sensitivity to stress exposure and GTN effects

In a first set of experiments, we validated the migraine model based on stress exposure followed by a subthreshold GTN dose. In CD-1 male mice, four consecutive days of restraint stress induced PMA in 85% of animals. Allodynia was evident one day after stress termination (1 DPS) and progressively declined over time, with nociceptive thresholds fully returning to baseline by 21 DPS. Administration of GTN (0.1 mg/kg, i.p.) at 21 DPS induced PMA exclusively in previously stressed mice, whereas no effect was observed in naïve animals. A different pattern was observed in females: although 4 days of restraint stress induced PMA that completely resolved by 21 DPS, GTN administration had no effect (Supplementary Figure 1). Based on these findings, subsequent experiments were carried out

in male mice only. Under the same experimental conditions, the phenotype of NOP(+/+) and NOP(–/–) mice was evaluated in parallel. No differences between genotypes were observed, either in the proportion of animals susceptible to stress-induced PMA, or in the time course of stress effects (Figure 2A), or in the sensitivity to GTN on 21 DPS (Figure 2B).

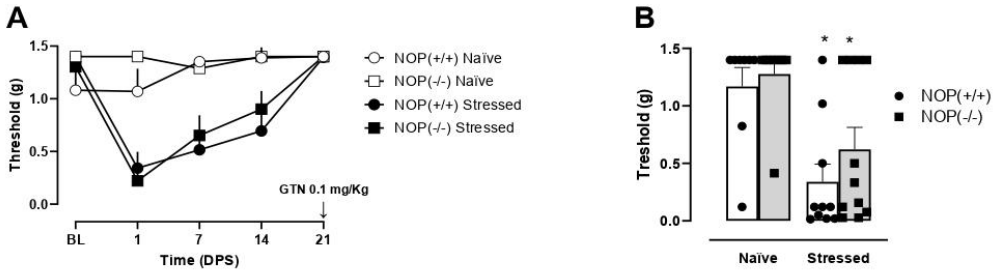

Figure 2. Phenotype of NOP(+/+) and NOP(–/–) mice in a migraine model induced by stress followed by GTN administration. (A) Time course of stress-induced changes in periorbital mechanical threshold. (B) Effect of a single GTN administration (0.1 mg/kg, i.p.) on periorbital mechanical threshold, measured 60 min post-injection. Two-way ANOVA (stress × genotype) followed by uncorrected Fisher's LSD revealed an effect of stress ($F(1,33) = 19.33$). *$p < 0.05$ vs naïve. Group sizes: NOP(+/+) naïve, N = 8; NOP(+/+) stressed, N = 10; NOP(–/–) naïve, N = 8; NOP(–/–) stressed, N = 11. BL, baseline; DPS, days post stress.

3.2. NOP agonists prevented GTN-induced PMA

The effects of NOP agonists were then assessed in the stress + GTN model of migraine in CD-1 mice. As expected, restraint stress induced PMA that spontaneously resolved by 21 DPS (Figure 3A). At this time point, compounds were administered 30 min before GTN (0.1 mg/kg, i.p.). Pretreatment with the brain-penetrant agonist AT-403 (30 µg/kg, i.p.) significantly attenuated GTN-induced PMA. Likewise, the peripherally restricted agonist UFP-112 (10 pmol, i.p.) also significantly reduced GTN-evoked PMA (Figure 3B).

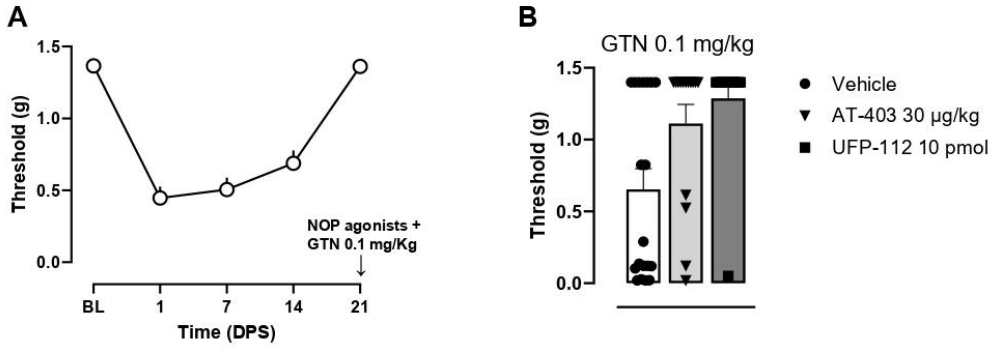

Figure 3. Effect of AT-403 and UFP-112 in a migraine model induced by stress followed by GTN administration. (A) Time course of stress-induced changes in periorbital mechanical threshold. (B) Effect of AT-403 and UFP-112 given i.p. 30 min before GTN (0.1 mg/kg, i.p.) administration on periorbital threshold, measured 60 min post GTN injection. One-way ANOVA followed by Tukey's multiple comparisons test revealed an effect of treatment ($F(2,40) = 7.28$). *$p < 0.05$ vs vehicle. Group sizes: vehicle, N = 19; AT-403, N = 12; UFP-112, N = 12. BL, baseline; DPS, days post stress.

3.3. NOP agonists reversed stress-induced PMA

An additional experiment was performed to determine whether NOP agonists were not only able to prevent pharmacologically induced PMA, but also capable of reversing stress-induced PMA. To this aim, compounds were administered at 1 DPS, when the periorbital mechanical threshold is maximally reduced, and their effects were monitored from 30 min post-injection up to 5 h. Both AT-403 and UFP-112 produced overlapping effects in terms of magnitude and time course. Notably, as early as 30 min after administration, both compounds fully reversed stress-induced PMA, and this effect persisted for at least 5 h (Figure 4).

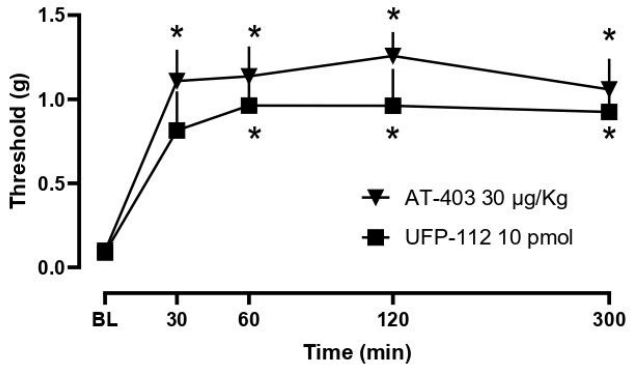

Figure 4. Effect of AT-403 and UFP-112 in a migraine model induced by stress. Effect of AT-403 and UFP-112 given i.p. on periorbital threshold, measured 30, 60, 120, and 300 min after NOP agonist treatment at 1 DPS. Two-way ANOVA followed by Tukey's multiple comparisons test revealed an effect of treatment ($F(2,40) = 7.28$). *$p < 0.05$ vs BL. Group sizes AT-403, N = 9; UFP-112, N = 9. BL, baseline.

A separate set of experiments was conducted in 9-month-old mice, an age group older than the 3–4-month-old animals used in the previous studies. In these mice, 4 days of restraint stress effectively induced PMA; however, unlike younger animals, no spontaneous recovery was observed. At 28 DPS, stressed mice still displayed significantly reduced thresholds compared to baseline (Figure 5A). This feature was exploited to test whether NOP agonists could reverse stress-induced PMA once it had become persistent. Both AT-403 and UFP-112 were administered at 28 DPS, and thresholds were assessed 2 h later. Remarkably, in this chronic condition both the brain-penetrant and the peripherally restricted agonist showed comparable efficacy in reversing stress-induced PMA (Figure 5B).

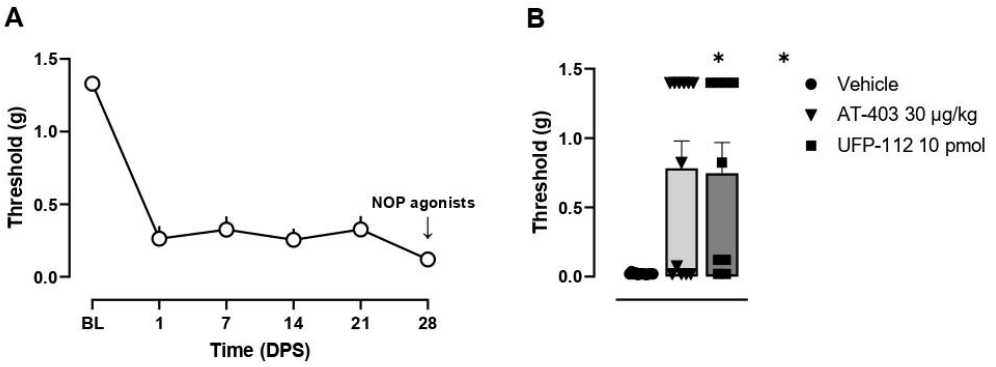

Figure 5. Effect of AT-403 and UFP-112 in a migraine model induced by stress. (A) Time course of stress-induced changes in periorbital mechanical threshold. (B) Effect of AT-403 and UFP-112 given i.p. on periorbital threshold, measured 120 min post NOP agonists injection. One-way ANOVA followed by Tukey's multiple comparisons test revealed an effect of treatment ($F(2,30) = 7.35$). *$p < 0.05$ vs vehicle. Group sizes: vehicle, N = 12; AT-403, N = 12; UFP-112, N = 9. BL, baseline; DPS, days post stress.

4. Discussion

In this study, we examined the effects of NOP receptor modulation in a mouse model of migraine induced by stress exposure and a subthreshold dose of GTN. Four days of restraint stress produced PMA in young mice (3–4 months), which spontaneously resolved within three weeks. In these animals, a subthreshold dose of GTN, inactive in naïve mice, was sufficient to induce PMA, indicating that stress primes susceptibility to normally innocuous migraine triggers. This finding is consistent with previous work showing that stress paradigms increase vulnerability to subthreshold stimuli such as NO donors or TRPA1 agonists [2,25,26,31,55]. The model therefore provides a tool to test genetic and pharmacological interventions under conditions relevant to migraine patients, where stress facilitates attacks triggered by otherwise harmless stimuli [13,43,51]. Notably, older mice (9 months) were more susceptible to stress, developing PMA that persisted for at least 28 days. This greater

vulnerability, in line with earlier reports on age-related stress sensitivity [6,32], offers an opportunity to investigate novel treatments in conditions that mimic chronic migraine.

Under the present experimental conditions, no differences were recorded between NOP(+/+) and NOP(-/-) mice, neither in terms of stress susceptibility, nor in terms of sensitivity to a subthreshold dose of GTN after stress sensitization. This result suggests that the endogenous N/OFQ does not participate in those mechanisms involving stress and GTN susceptibility under these experimental conditions. We recently reported no major differences between NOP(+/+) and NOP(-/-) mice in PMA evoked by CGRP [52], which is consistent with the lack of genotype-dependent differences in GTN responsiveness observed in the present study. Thus, it can be hypothesized that the endogenous N/OFQ – NOP system is not activated under the present conditions and does not interfere with the mechanisms underlying CGRP- or GTN-evoked PMA. On the other hand, a similar stress susceptibility of NOP(+/+) and NOP(-/-) mice is quite unexpected and does not align with previously reported studies. In fact, NOP(-/-) mice demonstrated less sensitivity to stress effects under different paradigms (i.e. forced swimming stress [19,22], tail suspension test [33], learned helplessness model [23,47], and corticosterone administration [11], suggesting the activation of the endogenous N/OFQergic system contributing to the deleterious effects of stress [17,18,42]. This has been confirmed using the NOPLight sensor, which enables the detection of N/OFQ in the ventral tegmental area of freely moving mice during stress exposure [63]. It is worth noting that all the above-mentioned studies addressed emotional (anxiety-like and depressive-like behaviors) and cognitive outcomes of stress, and this may explain the discrepancy between the results of the present study and the scientific literature. More aligned with the objectives of our study, the involvement of the N/OFQ / NOP system in stress-induced allodynia has been investigated by Zhang and colleagues using a single-prolonged stress model in rats, which results in persistent mechanical and thermal allodynia in the paw for up to 28 days. Pharmacological blockade of NOP reversed

stress-induced allodynia [62], while NOP(-/-) rats failed to develop allodynia after stress exposure [61], supporting a role for endogenous N/OFQ in mediating stress vulnerability. It should be noted, however, that Zhang's stress exposure procedures were considerably more severe than ours, and this, together with species differences, may account for the discrepancies between their findings and ours.

To investigate the effects of NOP activation in the stress + GTN migraine model, two chemically unrelated NOP agonists, AT-403 and UFP-112, were employed. AT-403 is a brain-penetrant small molecule. Its in vivo selectivity for the NOP receptor has already been demonstrated using NOP(-/-) mice [3]. AT-403 has been tested at a single dose of 30 µg/kg, which was selected based on a previous dose–response study demonstrating full efficacy in preventing CGRP-induced PMA [52]. UFP-112 is a potent, full agonist of the NOP receptor, whose in vivo pharmacological effects have been shown to rely exclusively on NOP activation, as confirmed by antagonist studies and experiments in NOP(-/-) mice [7,46]. The dose employed here was chosen according to our previously reported dose–response analysis [52]. Importantly, it has been demonstrated that even at doses 10,000-fold higher than the one used in this study, UFP-112 does not reach the brain, thus supporting its designation as a peripherally restricted agonist[52]. At the tested doses, neither AT-403 [3] nor UFP-112 [52] affects mouse locomotor activity.

Previous research shown the efficacy of NOP agonists in counteracting responses to migraine-producing substances. In particular, the NOP agonist Ro 64-6198, inhibited GTN-induced allodynia [53] and social deficit [35], while the analogue Ro 65-6570 reduced pain behaviors induced by the injection of formalin in the orofacial region [45]. Additionally, both the brain penetrant NOP agonists AT-403 and the peripherally restricted UFP-112 were effective in counteracting the PMA induced by CGRP, the most important migraine pain mediator [52]. Here, to increase the translational relevance, we moved to a different migraine paradigm, where PMA is induced by stress exposure and the

subsequent application of a subthreshold dose of GTN. This approach mimics the condition in migraine patients, where stress increases vulnerability to otherwise innocuous stimuli and facilitates attacks [13,43,51]. Under these experimental conditions, both AT-403 and UFP-112 displayed the same efficacy both in preventing the PMA induced by GTN on 21 DPS, and in reversing the PMA induced by stress on the first day after the exposure. Thus, NOP receptor activation proved useful not simply in preventing the PMA induced by drugs but also in reversing PMA immediately after stress exposure and, later, in preventing increased vulnerability to migraine triggers. To date, NOP agonists have been evaluated exclusively in acute migraine models, and their efficacy in a chronic context has remained unexplored. In this regard, our findings are particularly noteworthy: in 9-month-old mice exposed to stress, in which allodynia failed to resolve and persisted for up to 28 days, a single injection of AT-403 or UFP-112 was sufficient to completely reverse PMA and produce prolonged anti-allodynic effects. These results strongly suggest that NOP agonists may hold therapeutic potential not only in acute but also in chronic migraine conditions. It is noteworthy that the efficacy of a peripherally restricted agonist allows to avoid central actions elicited by brain-penetrant NOP agonists, e.g. somnolence [56,57,59].

Although the present study did not directly address the mechanisms through which NOP agonists counteract migraine, some speculation can be made based on the existing literature. The effectiveness of UFP-112 points toward the involvement of peripheral mechanisms. A substantial body of preclinical and clinical evidence implicates CGRP as a key mediator in migraine pathophysiology [14]. The pharmacokinetic profile of anti-CGRP agents, such as monoclonal antibodies [27,38], indicates that the peptide primarily exerts its pronociceptive actions in the periphery. More recently, it has been shown that in mice, CGRP contributes to the maintenance of PMA by engaging the CLR/RAMP1 receptor complex located in Schwann cells surrounding trigeminal cutaneous fibers [12]. Our previous work demonstrated that NOP receptor activation in Schwann cells can inhibit CGRP-induced rise in cAMP, and we proposed that

this functional antagonism may account for the ability of NOP agonists to reverse CGRP-induced PMA. Similar mechanisms are likely to be involved in the present migraine model. Avona et al. [2] demonstrated that a monoclonal antibody against CGRP completely abolished PMA induced by stress combined with a subthreshold dose of an NO donor, indicating that peripheral CGRP contributes to NO-induced responses in the primed state. In addition, Kopruszinski et al. [31] reported that, in a pilot study, restraint stress increased circulating CGRP levels in mice. On this basis, it can be hypothesized that NOP agonists exert their protective and therapeutic effects by counteracting CGRP signalling in Schwann cells.

The primary limitation of this study is its reliance on male mice only, even though migraine is a highly prevalent condition in females. This choice was made because, under our experimental conditions, we were unable to achieve priming to the subthreshold effects of GTN in female mice, and therefore, we proceeded with males only. However, we have recently shown the absence of sex differences both in the sensitivity to CGRP of NOP (+/+) and NOP(–/–) mice and in the efficacy of the two compounds in preventing CGRP-induced allodynia [52]. Similarly, Ro 64-6198 alleviated allodynia and social deficits induced by GTN without major differences between male and female mice [35,53]. These findings suggest that sex does not influence the mechanisms through which NOP receptor activation counteracts migraine-like behaviors.

In conclusion, our findings demonstrate that NOP receptor agonists are effective in both preventing and reversing migraine-like pain induced by stress and subthreshold GTN. These results strengthen the rationale for developing peripherally acting NOP agonists as innovative drugs for migraine management.

5. CONFLICT OF INTEREST

NZ is an employee of Astraea Therapeutics. The remaining authors declare no conflict of interest.

7. ACKNOWLEDGMENT

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

8. REFERENCES

[1] Arduin M, Spagnolo B, Calò G, Guerrini R, Carrà G, Fischetti C, Trapella C, Marzola E, McDonald J, Lambert DG, Regoli D, Salvadori S. Synthesis and biological activity of nociceptin/orphanin FQ analogues substituted in position 7 or 11 with Calpha,alpha-dialkylated amino acids. Bioorg Med Chem 2007;15:4434–4443.

[2] Avona A, Mason BN, Lackovic J, Wajahat N, Motina M, Quigley L, Burgos-Vega C, Moldovan Loomis C, Garcia-Martinez LF, Akopian AN, Price TJ, Dussor G. Repetitive stress in mice causes migraine-like behaviors and calcitonin gene-related peptide-dependent hyperalgesic priming to a migraine trigger. Pain 2020;161:2539–2550.

[3] Azevedo Neto J, Ruzza C, Sturaro C, Malfacini D, Pacifico S, Zaveri NT, Calò G. Functional selectivity does not predict antinociceptive/locomotor impairing potencies of NOP receptor agonists. Front Neurosci 2021;15:657153.

[4] Bartsch T, Akerman S, Goadsby PJ. The ORL-1 (NOP1) receptor ligand nociceptin/orphanin FQ (N/OFQ) inhibits neurogenic dural vasodilatation in the rat. Neuropharmacology 2002;43:991–998.

[5] Briscini L, Corradini L, Ongini E, Bertorelli R. Up-regulation of ORL-1 receptors in spinal tissue of allodynic rats after sciatic nerve injury. Eur J Pharmacol 2002;447:59–65.

[6] Buchanan JB, Sparkman NL, Chen J, Johnson RW. Cognitive and neuroinflammatory consequences of mild repeated stress are exacerbated in aged mice. Psychoneuroendocrinology 2008;33:755–765.

[7] Calo' G, Rizzi A, Cifani C, Micioni Di Bonaventura MV, Regoli D, Massi M, Salvadori S, Lambert DG, Guerrini R. UFP-112 a potent and long-lasting agonist selective for the Nociceptin/Orphanin FQ receptor. CNS Neurosci Ther 2011;17:178–198.

[8] Capuano A, Currò D, Dello Russo C, Tringali G, Pozzoli G, Di Trapani G, Navarra P. Nociceptin (1-13)NH2 inhibits stimulated calcitonin-gene-related-peptide release from primary cultures of rat trigeminal ganglia neurones. Cephalalgia 2007;27:868–876.

[9] Capuano A, De Corato A, Tringali G, Currò D, Dello Russo C, Navarra P. Buprenorphine inhibits bradykinin-induced release of calcitonin gene-related peptide from rat trigeminal neurons via both mu-opioid and nociceptin/orphanin peptide receptors. Eur J Pharmacol 2009;609:45–50.

[10] Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL. Quantitative assessment of tactile allodynia in the rat paw. J Neurosci Methods 1994;53:55–63.

[11] D'Oliveira da Silva F, Robert C, Lardant E, Pizzano C, Bruchas MR, Guiard BP, Chauveau F, Moulédous L. Targeting Nociceptin/Orphanin FQ receptor to rescue cognitive symptoms in a mouse neuroendocrine model of chronic stress. Mol Psychiatry 2024;29:718–729.

[12] De Logu F, Nassini R, Hegron A, Landini L, Jensen DD, Latorre R, Ding J, Marini M, Souza Monteiro de Araujo D, Ramírez-Garcia P, Whittaker M, Retamal J, Titiz M, Innocenti A, Davis TP, Veldhuis N, Schmidt BL, Bunnett NW, Geppetti P. Schwann cell endosome CGRP signals elicit periorbital mechanical allodynia in mice. Nat Commun 2022;13:646.

[13] Dresler T, Caratozzolo S, Guldolf K, Huhn J-I, Loiacono C, Niiberg-Pikksööt T, Puma M, Sforza G, Tobia A, Ornello R, Serafini G, European Headache Federation School of Advanced Studies (EHF-SAS). Understanding the nature of psychiatric comorbidity in migraine: a systematic review focused on interactions and treatment implications. J Headache Pain 2019;20:51.

[14] Edvinsson L, Haanes KA, Warfvinge K, Krause DN. CGRP as the target of new migraine therapies - successful translation from bench to clinic. Nat Rev Neurol 2018;14:338–350.

[15] Ferrari F, Malfacini D, Journigan BV, Bird MF, Trapella C, Guerrini R, Lambert DG, Calo' G, Zaveri NT. In vitro pharmacological characterization of a novel unbiased NOP receptor-selective nonpeptide agonist AT-403. Pharmacol Res Perspect 2017;5.

[16] Flores CA, Wang XM, Zhang KM, Mokha SS. Orphanin FQ produces gender-specific modulation of trigeminal nociception: behavioral and electrophysiological observations. Neuroscience 2001;105:489–498.

[17] Gavioli EC, Holanda VAD, Calo G, Ruzza C. Nociceptin/orphanin FQ receptor system blockade as an innovative strategy for increasing resilience to stress. Peptides 2021;141:170548.

[18] Gavioli EC, Holanda VAD, Ruzza C. NOP ligands for the treatment of anxiety and mood disorders. Handb Exp Pharmacol 2019;254:233–257.

[19] Gavioli EC, Marzola G, Guerrini R, Bertorelli R, Zucchini S, De Lima TCM, Rae GA, Salvadori S, Regoli D, Calo G. Blockade of nociceptin/orphanin FQ-NOP receptor signalling produces antidepressant-like effects: pharmacological and genetic evidences from the mouse forced swimming test. Eur J Neurosci 2003;17:1987–1990.

[20] Guerrini R, Calo G, Rizzi A, Bianchi C, Lazarus LH, Salvadori S, Temussi PA, Regoli D. Address and message sequences for the nociceptin receptor: a structure-activity study of nociceptin-(1-13)-peptide amide. J Med Chem 1997;40:1789–1793.

[21] Hawes BE, Graziano MP, Lambert DG. Cellular actions of nociceptin: transduction mechanisms. Peptides 2000;21:961–967.

[22] Holanda VAD, Oliveira MC, Da Silva Junior ED, Calo' G, Ruzza C, Gavioli EC. Blockade of nociceptin/orphanin FQ signaling facilitates an active copying strategy due to acute and repeated stressful stimuli in mice. Neurobiol Stress 2020;13:100255.

[23] Holanda VAD, Pacifico S, Azevedo Neto J, Finetti L, Lobão-Soares B, Calo G, Gavioli EC, Ruzza C. Modulation of the NOP receptor signaling affects resilience to acute stress. J Psychopharmacol (Oxford) 2019;33:1540–1549.

[24] Hou M, Uddman R, Tajti J, Edvinsson L. Nociceptin immunoreactivity and receptor mRNA in the human trigeminal ganglion. Brain Res 2003;964:179–186.

[25] Hu Y-Y, Mei H-R, Sankar S, Pirwani A, Akopian A, McIntyre C, Dussor G. Post-stress modulation of the HPA and melanocortin systems alleviates migraine-like behaviors in mice. Cephalalgia 2025;45:3331024251352856.

[26] Hu Y-Y, Souza R, Muthuraman A, Knapp L, McIntyre C, Dussor G. Glucocorticoid signaling mediates stress-induced migraine-like behaviors in a preclinical mouse model. Cephalalgia 2024;44:3331024241277941.

[27] Johnson KW, Morin SM, Wroblewski VJ, Johnson MP. Peripheral and central nervous system distribution of the CGRP neutralizing antibody [125I] galcanezumab in male rats. Cephalalgia 2019;39:1241–1248.

[28] Kelman L. The triggers or precipitants of the acute migraine attack. Cephalalgia 2007;27:394–402.

[29] Kiguchi N, Ding H, Kishioka S, Ko M-C. Nociceptin/Orphanin FQ Peptide Receptor-Related Ligands as Novel Analgesics. Curr Top Med Chem 2020;20:2878–2888.

[30] Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG. Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. PLoS Biol 2010;8:e1000412.

[31] Kopruszinski CM, Navratilova E, Swiokla J, Dodick DW, Chessell IP, Porreca F. A novel, injury-free rodent model of vulnerability for assessment of acute and preventive therapies reveals temporal contributions of CGRP-receptor activation in migraine-like pain. Cephalalgia 2021;41:305–317.

[32] Lee J-E, Kwon H-J, Choi J, Seo J-S, Han P-L. Aging increases vulnerability to stress-induced depression via upregulation of NADPH oxidase in mice. Commun Biol 2020;3:292.

[33] Medeiros IU, Ruzza C, Asth L, Guerrini R, Romão PRT, Gavioli EC, Calo G. Blockade of nociceptin/orphanin FQ receptor signaling reverses LPS-induced depressive-like behavior in mice. Peptides 2015;72:95–103.

[34] Meunier JC, Mollereau C, Toll L, Suaudeau C, Moisand C, Alvinerie P, Butour JL, Guillemot JC, Ferrara P, Monsarrat B. Isolation and structure of the endogenous agonist of opioid receptor-like ORL1 receptor. Nature 1995;377:532–535.

[35] Mudgal A, Wronikowska-Denysiuk O, Martinez M, Peters D, Maj M, Snow I, Pietrzak-Mitura D, Toll L, Ozawa A, Targowska-Duda KM. Ro 64-6198, a selective NOP receptor agonist attenuates social impairments associated with NTG-induced migraine pain. Neuropsychopharmacology 2025.

[36] Neal CR, Akil H, Watson SJ. Expression of orphanin FQ and the opioid receptor-like (ORL1) receptor in the developing human and rat brain. J Chem Neuroanat 2001;22:219–249.

[37] Nishi M, Houtani T, Noda Y, Mamiya T, Sato K, Doi T, Kuno J, Takeshima H, Nukada T, Nabeshima T, Yamashita T, Noda T, Sugimoto T. Unrestrained nociceptive response and disregulation of hearing ability in mice lacking the nociceptin/orphaninFQ receptor. EMBO J 1997;16:1858–1864.

[38] Noseda R, Schain AJ, Melo-Carrillo A, Tien J, Stratton J, Mai F, Strassman AM, Burstein R. Fluorescently-labeled fremanezumab is distributed to sensory and autonomic ganglia and the dura but not to the brain of rats with uncompromised blood brain barrier. Cephalalgia 2020;40:229–240.

[39] Ozawa A, Brunori G, Mercatelli D, Wu J, Cippitelli A, Zou B, Xie XS, Williams M, Zaveri NT, Low S, Scherrer G, Kieffer BL, Toll L. Knock-In Mice with NOP-eGFP Receptors Identify Receptor Cellular and Regional Localization. J Neurosci 2015;35:11682–11693.

[40] Pavelic AR, Wöber C, Riederer F, Zebenholzer K. Monoclonal Antibodies against Calcitonin Gene-Related Peptide for Migraine Prophylaxis: A Systematic Review of Real-World Data. Cells 2022;12.

[41] Pellesi L, Do TP, Hougaard A. Pharmacological management of migraine: current strategies and future directions. Expert Opin Pharmacother 2024;25:673–683.

[42] Pola P, Frezza A, Gavioli EC, Calò G, Ruzza C. Effects of Stress Exposure to Pain Perception in Pre-Clinical Studies: Focus on the Nociceptin/Orphanin FQ-NOP Receptor System. Brain Sci 2024;14.

[43] Rafique N, Al-Asoom LI, Latif R, Alsunni AA, Salem AM, Alkhalifa ZH, Almaharfi RM, Alramadan RS, Aldajani ZF, Alghadeer FAT, Albaghli LA. Prevalence of Migraine and its Relationship with Psychological Stress and Sleep Quality in Female University Students in Saudi Arabia. J Pain Res 2020;13:2423–2430.

[44] Reinscheid RK, Nothacker HP, Bourson A, Ardati A, Henningsen RA, Bunzow JR, Grandy DK, Langen H, Monsma FJ, Civelli O. Orphanin FQ: a neuropeptide that activates an opioidlike G protein-coupled receptor. Science 1995;270:792–794.

[45] Rizzi A, Ruzza C, Bianco S, Trapella C, Calo' G. Antinociceptive action of NOP and opioid receptor agonists in the mouse orofacial formalin test. Peptides 2017;94:71–77.

[46] Rizzi A, Spagnolo B, Wainford RD, Fischetti C, Guerrini R, Marzola G, Baldisserotto A, Salvadori S, Regoli D, Kapusta DR, Calo G. In vitro and in vivo studies on UFP-112, a novel potent and long lasting agonist selective for the nociceptin/orphanin FQ receptor. Peptides 2007;28:1240–1251.

[47] Silva AI, Holanda VAD, Azevedo Neto JG, Silva Junior ED, Soares-Rachetti VP, Calo G, Ruzza C, Gavioli EC. Blockade of NOP receptor modulates anxiety-related behaviors in mice exposed to inescapable stress. Psychopharmacology (Berl) 2020;237:1633–1642.

[48] Son H, Zhang Y, Shannonhouse J, Gomez R, Kim YS. PACAP38/mast-cell-specific receptor axis mediates repetitive stress-induced headache in mice. J Headache Pain 2024;25:87.

[49] Steiner TJ, Stovner LJ, Jensen R, Uluduz D, Katsarava Z, Lifting The Burden: the Global Campaign against Headache. Migraine remains second among the world's causes of disability, and first among young women: findings from GBD2019. J Headache Pain 2020;21:137.

[50] Stewart WF, Wood C, Reed ML, Roy J, Lipton RB, AMPP Advisory Group. Cumulative lifetime migraine incidence in women and men. Cephalalgia 2008;28:1170–1178.

[51] Stubberud A, Buse DC, Kristoffersen ES, Linde M, Tronvik E. Is there a causal relationship between stress and migraine? Current evidence and implications for management. J Headache Pain 2021;22:155.

[52] Sturaro C, Pola P, Argentieri M, Frezza A, Marini M, De Logu F, Albanese V, Soukupova M, Malfacini D, Zaveri NT, Nassini R, Jensen DD, Geppetti P, Calò G, Ruzza C. Activation of peripheral NOP receptors reduces periorbital mechanical allodynia evoked by CGRP in mice. Br J Pharmacol 2025.

[53] Targowska-Duda KM, Ozawa A, Bertels Z, Cippitelli A, Marcus JL, Mielke-Maday HK, Zribi G, Rainey AN, Kieffer BL, Pradhan AA, Toll L. NOP receptor agonist attenuates nitroglycerin-induced migraine-like symptoms in mice. Neuropharmacology 2020;170:108029.

[54] Wang XM, Zhang KM, Mokha SS. Nociceptin (orphanin FQ), an endogenous ligand for the QRL1 (opioid-receptor-like1) receptor; modulates responses of trigeminal neurons evoked by excitatory amino acids and somatosensory stimuli. J Neurophysiol 1996;76:3568–3572.

[55] Watanabe M, Kopruszinski CM, Moutal A, Ikegami D, Khanna R, Chen Y, Ross S, Mackenzie K, Stratton J, Dodick DW, Navratilova E, Porreca F. Dysregulation of serum prolactin links the hypothalamus with female nociceptors to promote migraine. Brain 2022;145:2894–2909.

[56] Whiteside GT, He E, Manjunath SS, Zhou M, Ahmad M, Harris SC. Efficacy and safety of a selective partial agonist for nociception/orphanin-FQ peptide (NOP) receptors in patients with insomnia disorder. Br J Clin Pharmacol 2025.

[57] Whiteside GT, Kyle DJ, Kapil RP, Cipriano A, He E, Zhou M, Shet MS, Hummel M, Knappenberger T, Fukumura K, Matsuo Y, Uehira M, Hiroyama S, Takai N, Willsie SK, Harris

SC. The nociceptin/orphanin FQ receptor partial agonist sunobinop promotes non-REM sleep in rodents and patients with insomnia. J Clin Invest 2024;134.

[58] Witta J, Palkovits M, Rosenberger J, Cox BM. Distribution of nociceptin/orphanin FQ in adult human brain. Brain Res 2004;997:24–29.

[59] Woodcock A, McLeod RL, Sadeh J, Smith JA. The efficacy of a NOP1 agonist (SCH486757) in subacute cough. Lung 2010;188 Suppl 1:S47-52.

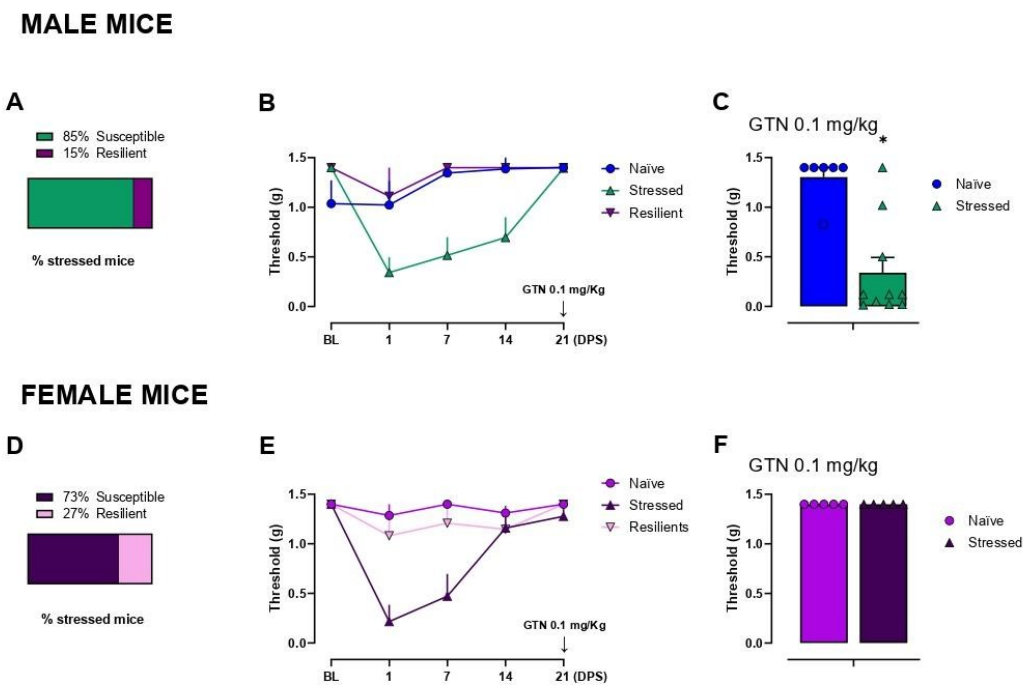
[60] Xie GX, Meuser T, Pietruck C, Sharma M, Palmer PP. Presence of opioid receptor-like (ORL1) receptor mRNA splice variants in peripheral sensory and sympathetic neuronal ganglia. Life Sci 1999;64:2029–2037.

[61] Zhang Y, Schalo I, Durand C, Standifer KM. Sex Differences in Nociceptin/Orphanin FQ Peptide Receptor-Mediated Pain and Anxiety Symptoms in a Preclinical Model of Post-traumatic Stress Disorder. Front Psychiatry 2018;9:731.

[62] Zhang Y, Simpson-Durand CD, Standifer KM. Nociceptin/orphanin FQ peptide receptor antagonist JTC-801 reverses pain and anxiety symptoms in a rat model of post-traumatic stress disorder. Br J Pharmacol 2015;172:571–582.

[63] Zhou X, Stine C, Prada PO, Fusca D, Assoumou K, Dernic J, Bhat MA, Achanta AS, Johnson JC, Pasqualini AL, Jadhav S, Bauder CA, Steuernagel L, Ravotto L, Benke D, Weber B, Suko A, Palmiter RD, Stoeber M, Kloppenburg P, Brüning JC, Bruchas MR, Patriarchi T. Development of a genetically encoded sensor for probing endogenous nociceptin opioid peptide release. Nat Commun 2024;15:5353.

Supplementary Figure 1



Behavior of male and female CD-1 mice in a migraine model induced by stress followed by GTN administration. (A) and (D) Percentage of mice developing PMA after 4 consecutive days of restraint stress (2 h/day). A mouse was considered susceptible when its periorbital mechanical threshold was below 0.4 g in at least one of the measurements at 1, 7, or 14 DPS. (B) and (E)Time course of stress-induced changes in periorbital mechanical threshold. (C) Effect of a single GTN administration (0.1 mg/kg, i.p.) on periorbital mechanical threshold, measured 60 min post-injection. *$p < 0.05$ vs naïve, Student's T test. BL, baseline; DPS, days post stress.

2.3 Synthesis and Pharmacological Characterization of Nociceptin/Orphanin FQ Dimeric Ligands

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Article

Synthesis and Pharmacological Characterization of Nociceptin/Orphanin FQ Dimeric Ligands

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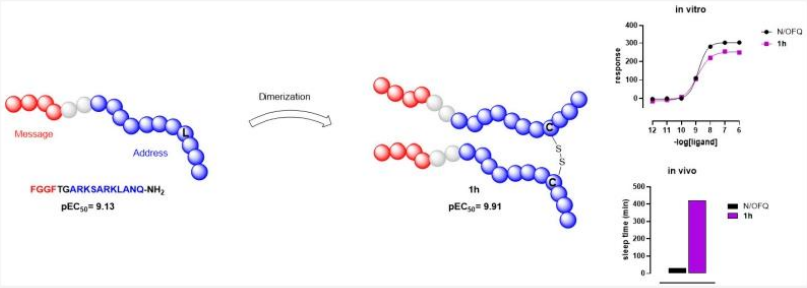

ABSTRACT: The neuropeptide nociceptin/orphanin FQ (N/OFQ) plays a key role in regulating several physiological functions and pathological states, which makes its receptor (NOP) a promising target for therapeutic interventions. In this study, we synthesized homodimeric N/OFQ-NH_2 derivatives linked by disulfide bonds between cysteines appropriately introduced in the addressing region of the native peptide in place of the original amino acids. The *in vitro* activity of the compounds was evaluated using both an NOP-G protein interaction BRET assay and a calcium mobilization assay. The most potent compound, **1h** (pEC_{50} > 9), was obtained by coupling two monomeric precursors via a Leu^{14}-to-Cys substitution. *In vivo*, **1h** demonstrated 3-fold greater potency than N/OFQ in eliciting loss of the righting reflex in mice and produced a long-lasting effect monitored for up to 7 h, supporting multimerization as a viable approach to developing long-acting peptide-based NOP ligands.

INTRODUCTION

The nociceptin/orphanin FQ (N/OFQ) peptide receptor (NOP) is a class A G protein-coupled receptor (GPCR) that specifically binds the endogenous neuropeptide N/OFQ.[1,2] Although classified within the opioid receptor family and sharing about 60% sequence homology with the classical opioid receptors (μ, δ, κ), the NOP receptor exhibits distinct pharmacological properties and ligand selectivity, reflecting unique features.[3] N/OFQ is a heptadecapeptide (FGGFTG-ARKSARKLANQ) that combines a "message" domain (Phe^1-Phe^4), which is essential for receptor activation, and a C-terminal "address" region (Ala^7-Gln^{17}) that plays a key role in enhancing binding affinity and receptor selectivity.[4,5] Initial X-ray crystallography studies[6,7] provided the first structural insights into the inactive, antagonist-bound state identifying a ligand-binding pocket formed by transmembrane domains TM3, TM5, TM6, and TM7. Earlier work had already pointed to extracellular loop 2 (ECL2) as a critical element in receptor activation mechanisms.[8] Recent cryo-EM studies have elucidated central structural features governing N/OFQ binding to the NOP receptor, revealing discrete conformational arrangements compared to antagonist-bound states.[9] Upon activation, the NOP receptor couples to Gi/Go proteins, resulting in the inhibition of adenylate cyclase activity, activation of potassium conductance, and suppression of calcium channel function.[3] N/OFQ and its receptors are extensively distributed across the central nervous system (CNS), with high expression levels in neuronal circuits and regions that play key roles in a variety of functions. These

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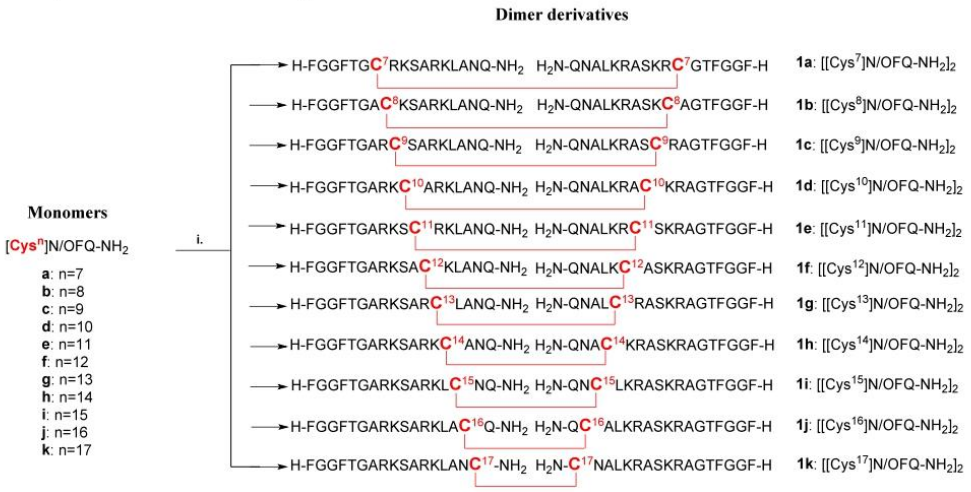
Scheme 1. Synthesis of Homodimeric Peptides 1a–k[a]



[a]Reagents and conditions: (i) $NaHCO_3$, H_2O/CH_3CN 1:1, 12 h, r.t.

include pain modulation, learning and memory, emotional regulation, stress responses, reward processing and substance abuse, neuroendocrine regulation, appetite control, and motor functions.[3] More recently, it has been demonstrated that the N/OFQ-NOP receptor system plays an important role in the control of the wake/sleep cycle.[10,11] Given its broad physiological relevance, the N/OFQ-NOP receptor system represents a promising therapeutic target for various pathological conditions. Several lines of evidence demonstrated that peptide[4] and nonpeptide[12] NOP receptor agonists produce potent analgesic effects by intrathecal delivery in nonhuman primates.[13] Moreover, mixed NOP/mu receptor agonists (i.e., cebranopadol) produce robust analgesic efficacy with reduced adverse effects typical of opioids.[14] Of note, NOP receptor agonists were shown to be potentially useful in managing anxiety disorders,[15] providing alternative options beyond current pharmacotherapies. In models of addiction, activation of the N/OFQ-NOP system has been shown to reduce drug-seeking behavior and attenuate withdrawal symptoms, highlighting its role in modulating reward pathways and stress responses.[16] Furthermore, NOP agonists have been investigated as potential hypnotics.[11,17]

At present, only two small molecule NOP receptor agonists, namely, cebranopadol and sunobinop, are undergoing clinical evaluation. Cebranopadol is a first-in-class oral mixed agonist of the NOP and classical opioid receptors and has advanced to phase III clinical trials for the treatment of moderate-to-severe pain.[18,19] Sunobinop, a NOP receptor selective partial agonist, is currently in phase II trials for insomnia related to alcohol cessation and alcohol use disorder.[11,20,21]

In addition to investigating non-peptide ligands for the NOP receptor, pharmaceutical research remains committed to developing stable and bioavailable peptide agonists, aiming to accelerate the translation of preclinical findings into innovative therapies. These efforts focus on optimizing the pharmacokinetic properties of the endogenous ligand while preserving its remarkable receptor affinity, selectivity, and efficacy. Herein, we describe our latest efforts in the development of N/OFQ oligomers derived from dimerization strategies of the native peptide. All compounds were evaluated *in vitro* in recombinant cells expressing the NOP receptor, using both a calcium mobilization assay performed on cells expressing the NOP receptor and chimeric G proteins[22] and a bioluminescence resonance energy transfer (BRET) assay to assess NOP–G protein interaction.[23] The most potent compound was further tested *ex vitro* in the electrically stimulated mouse vas deferens (mVD)[24] and, considering the known pro-hypnotic effects of NOP receptor agonists,[11,17,25,26] also evaluated *in vivo* for its ability to induce loss of the righting reflex (RR) in wild type and NOP knockout mice.

RESULTS

Design and Synthesis of N/OFQ-Related Dimeric Peptides. In our previous work,[27] we explored a series of homobivalent N/OFQ analogues by connecting the C-terminal of two N/OFQ(1–13)-NH_2 fragments (the minimal active sequence) using various spacers. Building on these findings, we investigated a new dimerization approach using the full-length native sequence of N/OFQ-NH_2. Specifically, we designed homodimer derivatives in which two N/OFQ-NH_2 molecules are linked together through disulfide bonds between cysteine residues strategically introduced at different positions of the address domain (see the structures of compounds **1a–k** shown in Scheme 1).

The dimerization reaction involved air oxidation in the presence of a catalytic amount of $NaHCO_3$ of the parent monomers ([Cys^n]N/OFQ-NH_2 with $n = 7$ to 17) dissolved in a 50% v/v mixture of H_2O and CH_3CN.[27] Each reaction was completed within 12 h, achieving full conversion of the monomers, simplifying the purification process in preparative HPLC. Dimers, after purification, showed a purity degree higher than 95% and were characterized by HRMS (HPLC and HRMS spectra reported in the Supporting Information).

***In Vitro* Studies.** The *in vitro* pharmacological activity of N/OFQ-NH_2 and its novel dimeric derivatives was evaluated using both a NOP-G protein interaction BRET assay[23] and a

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J. Med. Chem. XXXX, XXX, XXX–XXX

Table 1. Pharmacological Activities of N/OFQ Dimeric Analogues[a]

		NOP−G protein interaction		Ca^{2+} mobilization	
		pEC_{50} ($CL_{95\%}$)	E_{max} ± sem	pEC_{50} ($CL_{95\%}$)	E_{max} ± sem
	N/OFQ-NH_2	9.13 (8.67−9.60)	1.00	9.28 (9.02−9.54)	336 ± 16
1a	[[Cys^7]N/OFQ-NH_2]$_2$	8.71 (8.13−9.28)	1.09 ± 0.09	7.57 (7.09−8.05)	293 ± 18
1b	[[Cys^8]N/OFQ-NH_2]$_2$	8.04 (7.62−8.46)	1.04 ± 0.13	7.36 (6.42−8.30)	322 ± 20
1c	[[Cys^9]N/OFQ-NH_2]$_2$	8.87 (8.36−9.38)	1.03 ± 0.13	8.30 (7.90−8.70)	289 ± 18
1d	[[Cys^{10}]N/OFQ-NH_2]$_2$	9.85 (9.39−10.31)	1.07 ± 0.10	8.72 (8.28−9.17)	305 ± 42
1e	[[Cys^{11}]N/OFQ-NH_2]$_2$	9.38 (8.85−9.92)	1.06 ± 0.09	8.49(7.61−9.37)	290 ± 42
1f	[[Cys^{12}]N/OFQ-NH_2]$_2$	8.90 (8.45−9.34)	1.07 ± 0.12	8.52 (8.12−8.92)	346 ± 19
1g	[[Cys^{13}]N/OFQ-NH_2]$_2$	9.52 (8.75−10.29)	0.84 ± 0.06	8.79 (8.18−9.40)	247 ± 39
1h	[[Cys^{14}]N/OFQ-NH_2]$_2$	9.91 (9.14−10.68)	0.80 ± 0.08	9.14 (8.56−9.71)	241 ± 38
1i	[[Cys^{15}]N/OFQ-NH_2]$_2$	9.71 (8.95−10.48)	0.95 ± 0.03	8.53(7.78−9.28)	297 ± 26
1j	[[Cys^{16}]N/OFQ-NH_2]$_2$	9.76 (9.16−10.36)	0.98 ± 0.02	8.86 (8.35−9.37)	312 ± 38
1k	[[Cys^{17}]N/OFQ-NH_2]$_2$	9.71 (8.99−10.43)	0.97 ± 0.03	8.52 (8.36−8.69)	317 ± 29

[a] pEC_{50} values are expressed as mean ($CL_{95\%}$); E_{max} values are expressed as mean ± sem; N = 5 experiments performed in duplicate.

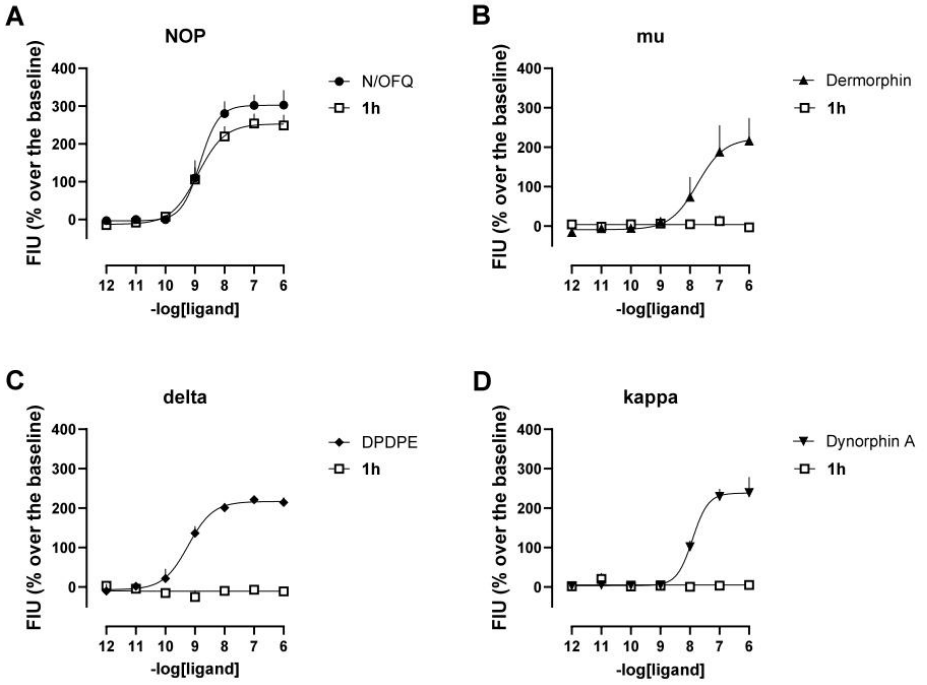

Figure 1. Compound **1h** selectivity of action at the NOP receptor in calcium mobilization experiments. Concentration−response curves to N/OFQ, dermorphin, DPDPE, and dynorphin A (i.e., standards) on CHO cells stably expressing the NOP (A), μ (B), δ (C), and κ (D) receptors and chimeric G proteins. Data are the mean ± sem of 5 experiments performed in duplicate.

calcium mobilization assay performed in cells expressing the recombinant NOP receptor together with a chimeric G protein that force the coupling to Ca^{2+} release (Table 1 and Supporting Information Figures S1 and S2).[22] N/OFQ-NH_2 induced concentration-dependent NOP/G-protein interaction and calcium release, with potency and maximal effects superimposable to those of the natural peptide N/OFQ (Supporting Information Figures S1 and S2). N/OFQ exhibited potencies and maximal responses consistent with previously published data.[22,23]

Regarding the NOP-G protein interaction, all the synthesized compounds demonstrated remarkable potency, with EC_{50} values falling within the subnanomolar to low single-digit nanomolar range. The highest potency (pEC_{50} = 9.91) was achieved by connecting two monomeric precursors via a leucine-to-cysteine substitution at position 14 of the native sequence (compound **1h**, [[Cys^{14}]N/OFQ-NH_2]$_2$). A slight but significant reduction in potency occurs when the linkage between the two N/OFQ chains is positioned closer to the message domain (see compounds **1a−c**). A comparable trend was observed at position 12, where substituting the arginine

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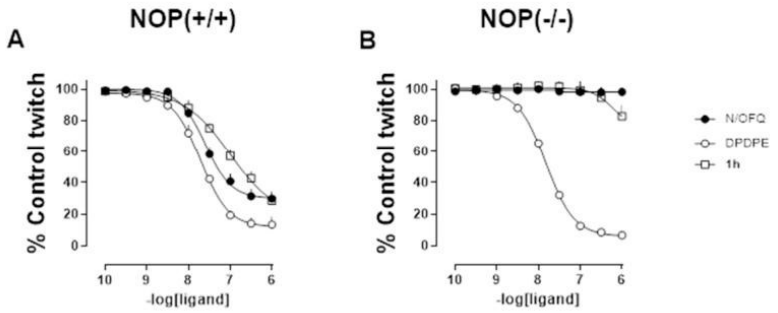

Figure 2. Mouse vas deferens bioassay. Concentration response curves to N/OFQ, DPDPE, and compound **1h** in NOP(+/+) (A) and NOP(−/−) (B) mice. Data are the mean ± sem of 5 experiments.

residue (critical for target binding)[4] with cysteine resulted in altered activity (compound **1f**).

In the calcium mobilization assay, the tested compounds confirmed nanomolar potency, albeit with slightly lower pEC_{50} values. Compound **1h** remained the most potent in the series, while a greater decrease in potency was observed when the branching is located near the message region. One-way ANOVA analysis revealed no statistically significant differences in E_{max} values among the compounds in either the NOP−G protein interaction or the calcium mobilization assay. Considering its promising profile *in vitro*, dimer **1h** was selected for further investigation.

Selectivity to the NOP receptor was carried out in cells expressing the μ, δ, and κ receptors and chimeric G proteins. Dermorphin, DPDPE ([D-Pen^2,D-Pen^5]enkephalin), and dynorphin A produced a robust concentration-dependent stimulation of calcium release in cells expressing μ, δ, and κ receptors, respectively, with high maximal effects and potency values. Compound **1h** was always inactive up to 1 μM (Figure 1), demonstrating more than 1000-fold selectivity for the NOP over classical opioid receptors.

In the electrically stimulated mVD bioassay, N/OFQ inhibited electrically induced contractions of tissues from wild-type mice (NOP(+/+)) in a concentration-dependent manner with a potency of 7.59 (7.41−7.77) and a maximal inhibition of 71 ± 4%. A comparable inhibitory effect was observed with the delta receptor agonist DPDPE, which showed a potency of 7.72 (7.45−7.99) and a maximal effect of 86 ± 6%. Compound **1h** mimicked the inhibitory action of N/OFQ, producing a similar maximal effect but with lower potency (pEC_{50} = 6.79 (6.02−7.56)) (Figure 2A). No significant differences in the kinetics of action between N/OFQ and compound **1h** were observed; both peptides induced rapid inhibition of the electrically evoked twitch, which was fully and rapidly reversible upon washout. To investigate the receptor(s) mediating the action of compound **1h** in the mVD, experiments were conducted using tissues from NOP knockout mice (NOP(−/−)). The concentration response curve to DPDPE was superimposable in tissues from both NOP(+/+) and NOP(−/−) mice. As expected, N/OFQ was completely inactive in tissues from NOP(−/−) animals (Figure 2B). Similarly, compound **1h** showed no effect on NOP(−/−) tissues.

***In Vivo* Studies.** Since it has been reported that N/OFQ and NOP receptor agonists can induce loss of the righting reflex (RR) in mice,[25,26] we evaluated the *in vivo* pharmacological activity of compound **1h** using this assay. The effects of **1h** were compared with those of its parent peptides N/OFQ and N/OFQ-NH_2. In addition, we included in the study a dimeric derivative of N/OFQ(1−13)-NH_2, previously reported by Pacifico et al. and referred to as compound **9** in the original publication,[27] and the tetrabranched N/OFQ derivative PWT2-N/OFQ (see Supporting Information Figure S3).[42] These compounds were tested in parallel with **1h** to investigate whether different dimerization/branching strategies may lead to distinct *in vivo* effects. At the high dose of 10 nmol, both N/OFQ and N/OFQ-NH_2 induced loss of the RR in fewer than half of the treated mice, with a short-lasting action (Figure 3A−C). In contrast, compound **9** induced RR loss in 62% of mice at 3 nmol and in all of the animals at 10 nmol (Figure 3D). In both cases, mice that lost the RR remained asleep until the experimental cutoff of 420 min (Figure 3F). PWT2-N/OFQ produced similar effects but was active starting from the dose of 0.3 nmol, which caused RR loss in 50% of treated mice (Figures 3G 3I). Results obtained with compound **1h** were similar to those of compound **9**; in fact, 75% of mice treated with 3 nmol and all mice treated with 10 nmol lost the RR, with a similarly prolonged effect lasting up to the 420 min cutoff (Figure 3L−N). These results suggest that N/OFQ dimerization enhances both *in vivo* potency and, more markedly, the duration of action, resulting in potent and long-lasting peptide agonists. However, no significant differences in *in vivo* activity were observed between the two dimerization strategies, leading to compound **9** and compound **1h**. The branching strategy used to obtain PWT2-N/OFQ appeared to confer slightly greater potency compared to the dimers, although no major differences were observed in terms of the duration of action. PWT2-N/OFQ was the only compound showing a significantly longer latency to RR loss compared to both N/OFQ and N/OFQ-NH_2. In contrast, compounds **9** and **1h** required a similar time from administration to RR loss, indicating that the enhanced duration of action observed with these dimers is not associated with delayed onset.

Finally, the selectivity of action of **1h** has been tested *in vivo* by treating NOP(−/−) mice with 10 nmol of the compound. Compound **1h** resulted completely inactive in NOP(−/−) mice (Figure 4A,B). On the contrary, diazepam (15 mg/kg, ip) produced similar hypnotic effects in NOP(+/+) and NOP(−/−) mice, suggesting a similar sensitivity of the two genotypes to classical hypnotic drugs (Figure 4C,D).

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J. Med. Chem. XXXX, XXX, XXX−XXX

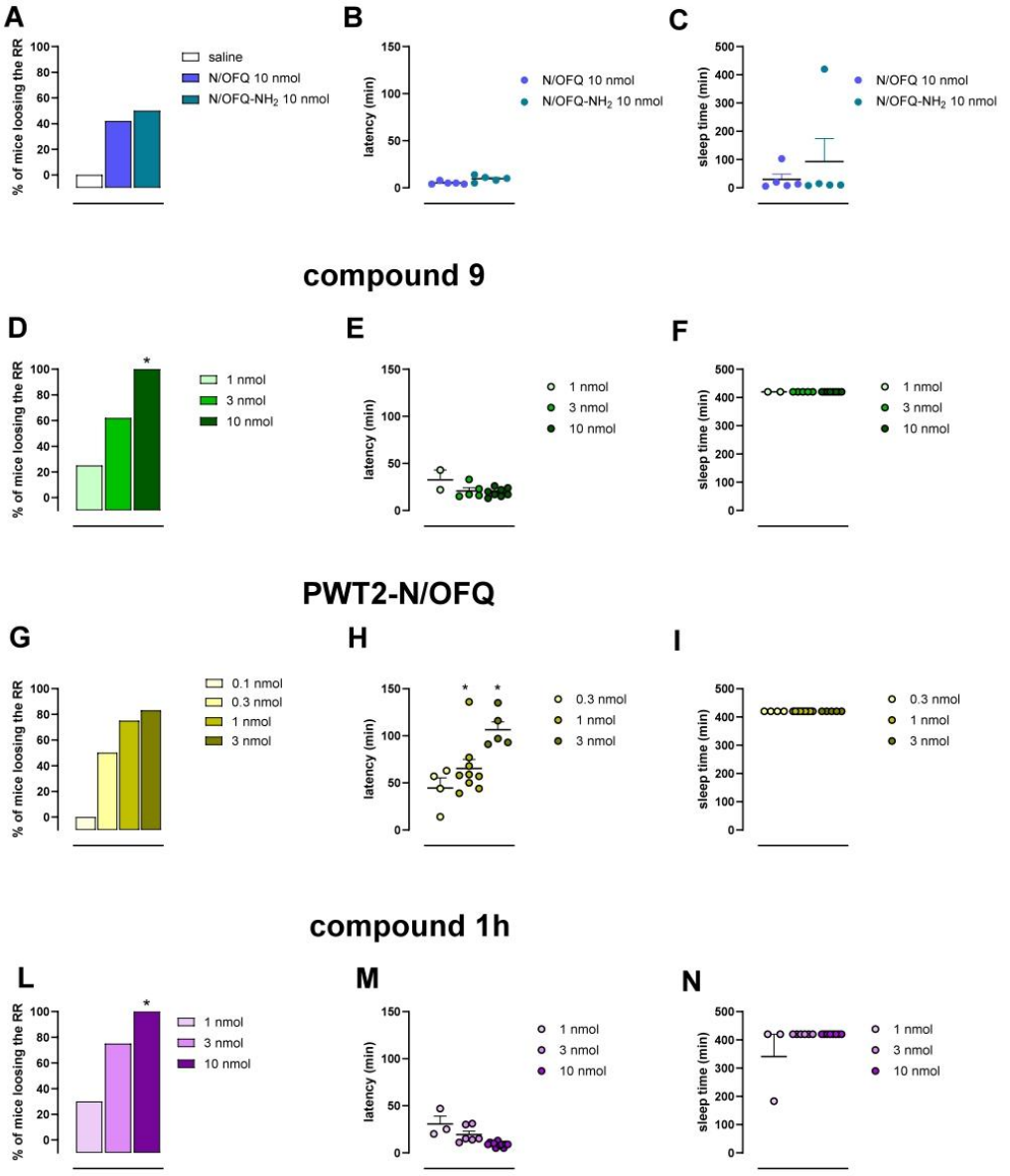

Figure 3. Loss of the RR assay. Panels A–C: N/OFQ and N/OFQ-NH_2 (10 nmol, icv). Panels D–F: compound **9**[27] (1–10 nmol, icv). Panels G–I: PWT2-N/OFQ[42] (0.1–3 nmol, icv). Panels L–N: compound **1h** (1–10 nmol, icv). Left panels show the percentage of animals that lost the RR; middle panels show the latency to lose the RR from compound injection; right panels show sleep duration. Sleep duration is defined as the interval between loss and recovery of the RR. Data are expressed as a percentage (RR loss) or mean and sem (latency and sleep duration). $N = 8–10$ mice per group; sleep-duration and latency graphs include only those animals that lost the RR. % of mice losing the RR: $^*p < 0.05$ vs N/OFQ-NH_2, according to Fisher's exact test. Latency: $^*p < 0.05$ vs N/OFQ-NH2, according to one-way ANOVA followed by Dunnet's post hoc test ($F(7,47) = 39.61$). Groups with N less than 5 were excluded from the analysis.

■ DISCUSSION

To overcome the typical limitations of therapeutic peptides, including poor stability *in vivo*, rapid enzymatic degradation, limited bioavailability, and difficulty in reaching target sites effectively, various approaches have been explored. Among these, peptide oligomerization has emerged as a particularly promising strategy aimed at enhancing the pharmacokinetic properties. For example, homobivalent peptide derivatives

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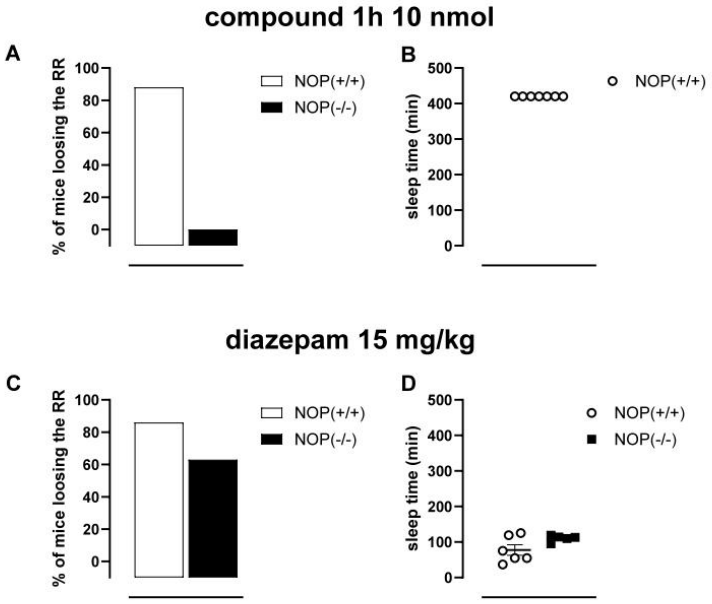

Figure 4. Loss of the RR assay. (A, B) Compound **1h** (10 nmol, icv) in NOP(+/+) and NOP(−/−) mice. (C, D) Diazepam (15 mg/kg, ip) in NOP(+/+) and NOP(−/−) mice. Left panels show the percentage of animals that lost the RR; right panels show their sleep duration. Sleep duration is defined as the interval between the loss and recovery of the RR. Data are expressed as a percentage (RR loss) or mean ± sem (sleep duration). N = 7−9 mice per group; sleep-duration graphs include only those animals that lost the RR.

have, in certain cases, shown a notable improvement in pharmacological properties over their monomeric counterparts, resulting in GPCR ligands with increased potency both *in vitro* and *in vivo*.[28−30] The enhanced biological activity seen in dimeric peptides is partly attributed to their capacity for multivalent interactions, allowing them to simultaneously bind multiple receptor sites. Over the past 40 years, extensive research on both peptide and nonpeptide multivalent ligands has provided strong evidence that many GPCRs exist not only as monomers but also as higher-order oligomers, particularly tetramers.[31] Multimeric constructs can indeed facilitate targeting of complex receptor systems, leading to more precise modulation of physiological responses and fine-tuning of receptor signaling pathways. Moreover, the greater steric hindrance resulting from dimerization can improve resistance to enzymatic degradation, thereby enhancing peptide stability and extending their half-life *in vivo*.[32−35] This may lead to clinical advantages such as longer lasting action and consequently less frequent dosing.

In the past, the multimerization approach has been explored to develop N/OFQ-related peptides with enhanced pharmacological properties. Particularly, the C-terminal portion of N/OFQ has been exploited for generating homo[27] and hetero[36−38] bivalent NOP ligands as well as homo[39] and hetero[40] tetrameric NOP ligands. While multiple lines of evidence have been gathered indicating that tetramerization of N/OFQ positively influences their half-life when administered *in vivo*,[4,39,41−43] the impact of dimerization on the duration of action of bioactive peptides remains less thoroughly investigated.

To these purposes, we recently investigated a series of homobivalent N/OFQ analogues developed by linking the C-terminal of two N/OFQ(1−13)-NH_2 units (as the minimal active sequence) via various spacers.[27] The study showed that *in vitro* activity remained mostly unchanged by ligand dimerization or variations in the spacer's length and composition. However, dimerization of the low-potency analogue N/OFQ(1−12)-NH_2 resulted in a complete restoration of potency in a mouse vas deferens assay. Similarly, when N/OFQ(1−12)-NH_2 was conjugated with the biologically inactive N/OFQ(2−12)-NH_2 (lacking the N-terminal F residue), the resulting heterodimer exhibited a potency comparable to that of N/OFQ(1−13)-NH_2.

These data suggested that dimerization at the address level, rather than at the message domain, is a key factor for the recognition and activation of the NOP receptor by the dimer peptide. The assumption prompted us to synthetise and investigate, both *in vitro* and *in vivo*, compounds **1a−k** described in this work and obtained through new dimerization strategies focused on the address domain.

For the *in vitro* characterization of all of the compounds, the G protein and calcium mobilization assays were used. These assays, differing in signal amplification, provide complementary information, allowing a more accurate evaluation of compound efficacy, which can vary depending on the system employed.[44] All of the synthesized compounds behaved as potent full agonists of the NOP receptor. The most potent compound was obtained by linking two monomeric precursors via cysteine residues at the 14-position (compound **1h**, [[Cys^{14}]N/OFQ-NH_2]$_2$). A slight loss in potency was observed when the branching was positioned close to the massage domain (see compounds **1a−c**) or when it required the replacement of amino acids important for target binding such as the positively charged Arg^{12} (compound **1f**).[4] In the case of **1h**, the insertion

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J. Med. Chem. XXXX, XXX, XXX−XXX

of a cysteine at position 14 replaces a leucine residue in the native sequence nonessential for biological activity.[45] Given its high potency, the pharmacological profile of compound **1h** was further investigated in the electrically stimulated mVD assay, a well-known N/OFQ sensitive pharmacological preparation, that offers the opportunity to characterize the pharmacological activity of NOP ligands at the murine receptor in its native environment.[24,46] Compound **1h** acted as a full NOP agonist, slightly less potent than N/OFQ and exhibited a rapid onset of action and full reversibility upon washout, mimicking the kinetic behavior of the natural ligand. Comparison with previously reported N/OFQ dimeric/branched derivatives, such as compound **9**[27] and the tetrabranched PWT2-N/OFQ,[42] reveals some differences. While compound **9** and PWT2-N/OFQ showed a modest increase in potency, **1h** displayed a ∼3-fold decrease compared to that of N/OFQ. Although we cannot currently explain this divergence, these variations are relatively minor, supporting the general observation that dimerization or branching has only a limited impact on NOP receptor activation *in vitro*. More notable are the kinetic differences. PWT2-N/OFQ showed a slow and poorly reversible interaction with the receptor,[42] suggesting that extensive branching may interfere with binding and dissociation kinetics. In contrast, both compound **9**[27] and **1h** maintained rapid and reversible kinetics, implying that dimerization does not significantly affect the binding dynamics. Finally, we assessed the selectivity of compound **1h** through calcium mobilization assays in cells expressing recombinant μ, δ, and κ opioid receptors as well as in mVD preparations from NOP(−/−) mice. In both systems, **1h** was inactive at the concentrations tested, demonstrating high selectivity for the NOP receptor. This contrasts with PWT2-N/OFQ, which, although inactive in recombinant cell lines, elicited weak effects in NOP(−/−) tissues at high concentrations, suggesting some loss of selectivity.[42] Overall, compound **1h** shows an improved selectivity profile relative to PWT2-N/OFQ. Encouraged by these results, **1h** was examined for its potential to induce a loss of the RR in mice. Compound **9** was included in the study as an example of a linear dimeric derivative with *in vitro* potency comparable to that of **1h**, albeit synthesized through a different fusion approach.[27] The aim was to compare the *in vivo* profile of a branched peptide dimer (**1h**) with that of a linear congener (**9**). The tetrameric derivative PWT2-N/OFQ has been included in the study to test whether tetramerization may offer an advantage over dimerization. While the pharmacological activity of compound **9** has so far been characterized only *in vitro*, PWT2-N/OFQ has been extensively studied *in vivo* across various assays and always in comparison with N/OFQ. These comprehensive *in vivo* studies consistently demonstrated that PWT2-N/OFQ mimicked the actions of the natural peptide N/OFQ but was more potent (approximately 30-fold[41−43]), elicited larger effects (e.g., ref 42), and exhibited a remarkably prolonged duration of action.[43] Of note, no *in vivo* selectivity issues were detected for PWT2-N/OFQ. Both NOP(−/−) and antagonism studies robustly demonstrated that its effects are solely due to selective NOP receptor activation.[41,43] Here compound **1h** resulted 3-fold more potent than N/OFQ and N/OFQ-NH_2 in inducing loss of the RR reflex in mice. Moreover, while in those mice that lost the RR N/OFQ and N/OFQ-NH_2 effects lasted only few minutes, **1h** produced a long-lasting effect that reached the experimental cutoff of 7 h. Importantly, **1h** was completely inactive in NOP(−/−) mice, demonstrating that the mechanism by which it induces the loss of RR is the selective activation of the NOP receptor. Of note, no differences in the sensitivity to the hypnotic effects of diazepam were measured between NOP(+/+) and NOP(−/−) mice, suggesting that the inactivity of **1h** in NOP(−/−) mice is not due to a reduced sensitivity to hypnotic substances of the mutant mice. Compound **9** displayed a similar *in vivo* activity to **1h**, in terms of both potency and duration of action. Thus, both dimerization approaches were useful to obtain more potent and longer-lasting peptides, with no significant advantages of one strategy over the other one. The tetrabranched peptide proved somewhat advantageous in terms of potency, being 10-fold more potent than compounds **9** and **1h** and, in line with previous studies,[41−43] 30 times more potent than N/OFQ. However, the duration of action was similarly high for both dimeric and tetra-branched derivatives (at least 40 times longer than that of N/OFQ), suggesting that neither multimerization strategy offers a clear superiority over the other. However, since all compounds reached the assay cutoff for duration of action, it is not possible to draw definitive comparisons between the three compound types. Regarding the onset of action, no statistically significant differences were observed among N/OFQ, N/OFQ-NH_2, compound **9**, and compound **1h**, all showing a rapid induction of the effect. In contrast, PWT2-N/OFQ required a longer time to induce the loss of the RR, a slow onset that is consistent with previous reports in the literature.[42] Thus, although tetramerization appears to be associated with a delayed onset of action, this was not observed with dimeric derivatives. What emerges from the *in vivo* results of this study is that icv administration of peptides with a longer duration of action compared to the very short-acting N/OFQ leads to a remarkably prolonged loss of the RR reflex in mice. This effect appears to last longer than what has been reported in the literature for nonpeptide NOP agonists such as Ro 64-6198[17] or Sunobinop,[11] which, at EEG recordings, induced sleep for approximately 3 to 5 h in rats. Although the methodologies differ and a direct comparison is not possible, the strikingly long-lasting RR suppression observed here remains difficult to explain. One possible explanation is that the central route of administration used in this study allows the peptides to reach very high local concentrations in specific brain regions, which may be less accessible when the compounds are administered systemically. However, this remains a working hypothesis, and further neurobiological investigations are needed to better define the mechanisms and neural circuits underlying the NOP agonist-induced sleep. Overall, what can be concluded from this assay is that all of the multimeric peptides tested here appeared to be long-acting. Future studies using alternative behavioral assays will be necessary to further clarify the pharmacokinetic properties and duration of action of these compounds *in vivo*.

■ CONCLUSIONS

In this study, we reported the discovery and characterization of novel peptide ligands targeting the NOP receptor, designed through a dimerization strategy of N/OFQ that led to the branching of the peptide address domain. The approach adopted provided the analogue **1h** that was 3-fold more potent than N/OFQ in inducing the loss of the RR reflex in mice, with a long-lasting effect monitored for 7 h. The comparison with the dimeric N/OFQ analogue compound **9** revealed that peptide dimerization, independent from the final architecture (linear or branched), is responsible for the significant increase

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J. Med. Chem. XXXX, XXX, XXX−XXX

in the *in vivo* duration of action. Contrary to peptide tetramerization as in PWT2-N/OFQ, dimerization does not significantly affect the NOP binding dynamics. Our findings contribute to the expanding repertoire of NOP receptor agonists and provide insights into the structural and functional advantages conferred by peptide dimerization, advancing the development of therapeutics targeting N/OFQ pathways.

MATERIALS AND METHODS

Solid Phase Peptide Synthesis. All monomers were synthesized via solid-phase peptide synthesis (SPPS) by using standard Fmoc/tBu chemistry. Fmoc-amino acids were purchased from BLDpharm, Zentek, and Sigma-Aldrich. Polystyrene resin functionalized with a Rink Amide AmphiSpheres 20 RAM linker (derivatization: 0.55 mmol/g; 75−150 μm) served as the solid support, enabling C-terminal amidation upon cleavage. Synthesis was performed on a SyroXP automatic peptide synthesizer. The protocol involved sequential cycles of coupling, capping, and Fmoc deprotection. Fixed concentrations of HBTU (0.62 M), DIPEA (0.87 M), Ac_2O (0.5 M), NMM (0.25 M), and piperidine (40%) in DMF were used for automated reaction cycles. Peptide cleavage from the solid support was achieved using a cleavage cocktail composed of TFA/triisopropylsilane/H_2O (95:2.5:2.5) at room temperature for 4 h (10 mL). The resin was removed by filtration, and the filtrate was first concentrated by rotary evaporation and then treated with Et_2O at −20 °C to precipitate the peptides. Finally, the precipitated peptides were centrifuged and dried. Crude reaction products were purified via a reverse-phase 1260 Infinity II preparative LC system equipped with a Jupiter (Phenomenex) C18, 15 μm, 300 Å column (250 mm × 30 mm). Elution was performed with a linear gradient of solvents A (100% H_2O and 0.1% TFA) and B (40% H_2O, 60% CH_3CN, and 0.1% TFA) at a flow rate of 20 mL/min. The gradient program was optimized based on the analytical HPLC profile of the crude peptide. The molecular weights of reaction intermediates and final products were determined by using an ESI (electrospray ionization) MICROMASS ZMD 2000 mass spectrometer. The purity of the final compounds was determined by analytical reverse-phase HPLC on an Agilent Technologies 1200 series system equipped with a UV detector. A Kinetex (Phenomenex) 5 μm C18 100 Å (150 mm × 4.6 mm) LC column was used, eluted with a polar mobile phase consisting of solvent A (100% H_2O, 0.1% TFA) and solvent B (100% CH_3CN, 0.1% TFA). A linear gradient from 100% A to 100% B was applied over 25 min. All final compounds were >95% pure by HPLC analysis (see Supporting Information for HPLC profiles). High resolution masses for the final compounds were determined on a Vanquish Flex UHPLC coupled to an Orbitrap Exploris 120 HRMS instrument (see Supporting Information).

General Procedure for the Synthesis of the Dimeric Peptides. To a solution of each monomer ([Cys^{tt}]N/OFQ-NH_2, 10 μmol) in a mixture of H_2O/CH_3CN (1:1, 1 mL), 5 μL of a 5% aqueous $NaHCO_3$ was added. The reaction progress was monitored via ESI-MS. After it was completed, the crude mixture was purified by preparative HPLC.

Calcium Mobilization Assay. Chinese hamster ovary (CHO) cells stably coexpressing the human NOP, μ, or κ opioid receptors and the $G\alpha_{qi5}$ protein or the human δ and the $G\alpha_{qG66Di5}$ protein were used in this assay.[22,47] Cells were cultured in DMEM/F-12 (1:1) medium supplemented with 10% FBS, 2 mM L-glutamine, 200 mg/mL G418, 100 mg/mL hygromycin B, 100 IU/mL penicillin, and 100 IU/mL streptomycin. Cells were maintained at 37 °C in a humidified atmosphere with 5% CO_2 and were seeded at 50 000 cells/well into 96-well black, clear-bottom plates 24 h before test. Loading for 45 min with a solution consisting of HBSS supplemented with 2.5 mM probenecid, 3 μM Fluo-4 AM, and 0.01% pluronic acid ensures the calcium-sensitive dye (Fluo-4) reaches the needed concentration inside the cell, while the exchange of the solution with 100 μL/well of buffer consisting of HBSS with 20 mM HEPES, 2.5 mM probenecid, and 500 μM Brilliant Black allows for fluorescence background to decrease. Serial dilutions of ligands were prepared in HBSS buffer with 20 mM HEPES and 0.02% bovine serum albumin (BSA) to minimize the ligands' stickiness to plasticware. The automated microplate reader FlexStation II (Molecular Devices, CA, US) was employed at 37 °C to detect changes in fluorescence intensity. The effects of all compounds were expressed as the maximum change in percentage over the baseline fluorescence measured in samples treated with vehicles.

NOP Receptor−G Protein Interaction. A BRET interaction assay was used, as previously detailed in ref 23, to study the propensity of ligands to evoke interaction between the NOP receptor and to G protein. Membranes taken from HEK293 cells stably coexpressing the fusoproteins NOP-RLuc and Gβ1-RGFP were used. Such cells were grown in DMEM medium supplemented with 10% FBS, 2 mM L-glutamine, 200 mg/mL G418, 100 mg/mL hygromycin B, 100 IU/mL penicillin, and 100 IU/mL streptomycin in a humidified atmosphere with 5% CO_2 at 37 °C. Cell membranes were thawed and resuspended in PBS supplemented with 0.01% BSA before the assay, and an amount of 3 μg of total protein was dispensed in each of the 96 wells together with 2 μM Prolume Purple Coelenterazine. All experiments were carried out at room temperature. Ligands were added, and averaged BRET ratios from 10 min measurements were computed. BRET ratios were computed as counts per second (CPS) obtained on a Victor Nivo (PerkinElmer) with 405 (10) nm and 510 (30) nm bandpass filters. Vehicles' BRET ratios were derived from light passed through 510 divided by that passed through 405 filters. Vehicles BRET values were subtracted from all computations, and data sets were normalized as a fraction of N/OFQ maximal effects.

Mouse Vas Deferens Bioassay. This study was approved by the Animal Welfare Body of the University of Ferrara and by the Italian Ministry of Health (authorization number CBCC2.N.BXI). Experiments were performed on an isolated mouse vas deferens. All experiments were conducted with mice bred and housed in the University of Ferrara's animal facility under specific pathogen-free conditions (SPF). All mice were housed in cages with individual ventilation, with a constant temperature of 21 °C, 60% humidity, and a 12 h light/dark cycle. Food and water were provided ad libitum. CD-1 NOP(+/+) and CD-1 NOP(−/−) male mice, aged 9 to 12 months, were used. Animals were sacrificed on the day of the experiment with CO_2 overdose. Bioassay experiments were performed as previously described.[24] The tissues were suspended in 5 mL organ bath containing Krebs solution (NaCl 118.5 nM, KCl 4.7 nM, KH_2PO_4 1.2 nM, $NaHCO_3$ 25 nM, $CaCl_2$ 2.5 nM, glucose 10 nM). The Krebs solution was oxygenated with 95% O_2 and 5% CO_2 and the temperature set at 33 °C with a resting tension of 0.3 g applied to the tissues. Tissues were stimulated through two platinum electrodes with a supramaximal rectangular pulse of 1 ms duration, 0.05 Hz frequency, and 80 V of amplitude. The electrically evoked contractions were measured isotonically by means of Basile strain gauge transducers (Basile, IT) and a recorder with a Power Lab 8 instrument (ADInstruments, CO, U.S.). Following an equilibration period of approximately 60 min, the contractions induced by electrical field stimulation were stable. At this time, cumulative concentration response curves were carried out.

Loss of the Righting Reflex Assay. All of the experimental procedures adopted in the *in vivo* studies comply with the European Directive 2010/63/EU on protecting animals used for scientific purposes and Italian Legislative Decree no. 26 of March 4, 2014. These experiments were approved by the Animal Welfare Body of the University of Ferrara and by the Ministry of Health (authorization number 677/2024-PR). *In vivo* studies have been reported following ARRIVE guidelines.[48] 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 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;[49,50] these mice have been backcrossed on CD-1 strain in our laboratories. NOP(+/+) and

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J. Med. Chem. XXXX, XXX, XXX−XXX

NOP(−/−) littermates were obtained by mating with NOP(±) mice. All mice were genotyped using the polymerase chain reaction previously described.[51] All mice were used only once. The RR assay was performed as previously described.[52] Mice were given an intracerebroventricular (icv) injection of saline, N/OFQ (10 nmol), N/OFQ-NH_2 (10 nmol), compound **9** (1−10 nmol),[27] PWT2-N/OFQ (0.1−3 nmol),[42] or compound **Ih** (1−10 nmol). Diazepam 15 mg/kg was injected ip. When the animals lost RR, they were placed in a plastic cage, and the time was recorded by an expert observer, blind to drug treatments and/or genotype. Animals were judged to have regained the RR response when they could correct themselves three times within 30 s. Sleeping time is defined as the amount of time between the loss and regaining of the RR; it was rounded to the nearest minute.

Data Analysis, Statistics, and Terminology. Concentration-response curves to agonists were analyzed by a four-parameter logistic nonlinear regression model: Effect = baseline + (E_{max} − baseline)/(1 + $10^{((\log EC_{50} - \log[ligand])slope)}$). In BRET the effects were normalized to that of N/OFQ-NH_2 (E_{max} = 1). Experimental data were expressed as mean ± sem of at least 5 experiments. Potency values were expressed as the mean and $CL_{95\%}$. *In vitro* E_{max} values were analyzed by one-way ANOVA, while *in vivo* latency data were evaluated by one-way ANOVA followed by Dunnett's post hoc test. The proportion of mice losing the righting reflex was compared by Fisher's exact test performed on raw data. GraphPad Prism 10.0 software was used for all of the analyses. The terminology employed is consistent with the International Union of Basic and Clinical Pharmacology (IUPHAR) recommendations.[53,54]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jmedchem.5c02350.

Figures S1 and S2 (NOP−G protein interaction assay and calcium mobilization assay); Figure S3 (chemical structure of compound **9** and PWT2-N/OFQ); HRMS spectra (pp S6−S16) and HPLC traces (pp S17−S22) of dimeric peptides **1a**−**k** (PDF)

Molecular formula strings and some data (CSV)

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Notes

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■ ABBREVIATIONS USED

BRET, bioluminescence resonance energy transfer; BSA, bovine serum albumin; CHO cells, Chinese hamster ovary cells; CL95%, 95% confidence limit; DIPEA, *N,N*-diisopropylethylamine; FBS, fetal bovine serum; HBSS, Hanks' balanced salt solution; HBTU, *N,N,N′,N′*-tetramethyl-*O*-(1*H*-benzotria-

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J. Med. Chem. XXXX, XXX, XXX−XXX

zol-1-yl)uronium hexafluorophosphate; mVD, mouse vas deferens; N/OFQ, nociceptin/orphanin FQ; NOP, nociceptin/orphanin FQ (N/OFQ) peptide receptor; PWT, peptide welding technology; RR, loss of the righting reflex; sem, standard error of the mean; SPPS, solid-phase peptide synthesis

■ REFERENCES

(1) Meunier, J. C.; Mollereau, C.; Toll, L.; Suaudeau, C.; Moisand, C.; Alvinerie, P.; Butour, J. L.; Guillemot, J. C.; Ferrara, P.; Monsarrat, B.; et al. Isolation and structure of the endogenous agonist of opioid receptor-like ORL1 receptor. *Nature* **1995**, *377* (6549), 532−535.

(2) Reinscheid, R. K.; Nothacker, H. P.; Bourson, A.; Ardati, A.; Henningsen, R. A.; Bunzow, J. R.; Grandy, D. K.; Langen, H.; Monsma, F. J., Jr; Civelli, O. Orphanin FQ: a neuropeptide that activates an opioidlike G protein-coupled receptor. *Science (New York, N.Y.)* **1995**, *270* (5237), 792−794.

(3) Toll, L.; Bruchas, M. R.; Calo', G.; Cox, B. M.; Zaveri, N. T. Nociceptin/Orphanin FQ Receptor Structure, Signaling, Ligands, Functions, and Interactions with Opioid Systems. *Pharmacol. Rev.* **2016**, *68* (2), 419−457.

(4) Preti, D.; Caló, G.; Guerrini, R. NOP-Targeted Peptide Ligands. *Handbook of experimental pharmacology* **2018**, *254*, 17−36.

(5) Guerrini, R.; Calo', G.; Rizzi, A.; Bianchi, C.; Lazarus, L. H.; Salvadori, S.; Temussi, P. A.; Regoli, D. Address and message sequences for the nociceptin receptor: a structure-activity study of nociceptin-(1-13)-peptide amide. *Journal of medicinal chemistry* **1997**, *40* (12), 1789−1793.

(6) Thompson, A. A.; Liu, W.; Chun, E.; Katritch, V.; Wu, H.; Vardy, E.; Huang, X. P.; Trapella, C.; Guerrini, R.; Calo, G.; Roth, B. L.; Cherezov, V.; Stevens, R. C. Structure of the nociceptin/orphanin FQ receptor in complex with a peptide mimetic. *Nature* **2012**, *485* (7398), 395−399.

(7) Miller, R. L.; Thompson, A. A.; Trapella, C.; Guerrini, R.; Malfacini, D.; Patel, N.; Han, G. W.; Cherezov, V.; Caló, G.; Katritch, V.; Stevens, R. C. The Importance of Ligand-Receptor Conformational Pairs in Stabilization: Spotlight on the N/OFQ G Protein-Coupled Receptor. *Structure* **2015**, *23* (12), 2291−2299.

(8) Mollereau, C.; Mouledous, L.; Lapalu, S.; Cambois, G.; Moisand, C.; Butour, J. L.; Meunier, J. C. Distinct mechanisms for activation of the opioid receptor-like 1 and kappa-opioid receptors by nociceptin and dynorphin A. *Molecular pharmacology* **1999**, *55* (2), 324−331.

(9) Wang, Y.; Zhuang, Y.; DiBerto, J. F.; Zhou, X. E.; Schmitz, G. P.; Yuan, Q.; Jain, M. K.; Liu, W.; Melcher, K.; Jiang, Y.; Roth, B. L.; Xu, H. E. Structures of the entire human opioid receptor family. *Cell* **2023**, *186* (2), 413−427.

(10) Morairty, S. R.; Sun, Y.; Toll, L.; Bruchas, M. R.; Kilduff, T. S. Activation of the nociceptin/orphanin-FQ receptor promotes NREM sleep and EEG slow wave activity. *Proc. Natl. Acad. Sci. U.S.A.* **2023**, *120* (13), No. e2214171120.

(11) Whiteside, G. T.; Kyle, D. J.; Kapil, R. P.; Cipriano, A.; He, E.; Zhou, M.; Shet, M. S.; Hummel, M.; Knappenberger, T.; Fukumura, K.; Matsuo, Y.; Uehira, M.; Hiroyama, S.; Takai, N.; Willsie, S. K.; Harris, S. C. The nociceptin/orphanin FQ receptor partial agonist sunobinop promotes non-REM sleep in rodents and patients with insomnia. *J. Clin. Invest.* **2024**, *134* (1), No. e171172.

(12) Zaveri, N. T.; Meyer, M. E. NOP-Targeted Nonpeptide Ligands. *Handbook of experimental pharmacology* **2019**, *254*, 37−67.

(13) Ding, H.; Kiguchi, N.; Dobbins, M.; Romero-Sandoval, E. A.; Kishioka, S.; Ko, M. C. Nociceptin Receptor-Related Agonists as Safe and Non-addictive Analgesics. *Drugs* **2023**, *83* (9), 771−793.

(14) Calo, G.; Lambert, D. G. Nociceptin/orphanin FQ receptor ligands and translational challenges: focus on cebranopadol as an innovative analgesic. *British journal of anaesthesia* **2018**, *121* (5), 1105−1114.

(15) Goeldner, C.; Spooren, W.; Wichmann, J.; Prinssen, E. P. Further characterization of the prototypical nociceptin/orphanin FQ peptide receptor agonist Ro 64-6198 in rodent models of conflict anxiety and despair. *Psychopharmacology* **2012**, *222* (2), 203−214.

(16) Lutfy, K.; Zaveri, N. T. The Nociceptin Receptor as an Emerging Molecular Target for Cocaine Addiction. *Progress in molecular biology and translational science* **2016**, *137*, 149−181.

(17) Morairty, S. R.; Sun, Y.; Toll, L.; Bruchas, M. R.; Kilduff, T. S. Activation of the nociceptin/orphanin-FQ receptor promotes NREM sleep and EEG slow wave activity. *Proc. Natl. Acad. Sci. U.S.A.* **2023**, *120* (13), No. e2214171120.

(18) Schunk, S.; Linz, K.; Hinze, C.; Frormann, S.; Oberbörsch, S.; Sundermann, B.; Zemolka, S.; Englberger, W.; Germann, T.; Christoph, T.; Kögel, B. Y.; Schröder, W.; Harlfinger, S.; Saunders, D.; Kless, A.; Schick, H.; Sonnenschein, H. Discovery of a Potent Analgesic NOP and Opioid Receptor Agonist: Cebranopadol. *ACS medicinal chemistry letters* **2014**, *5* (8), 857−862.

(19) Rizzi, A.; Cerlesi, M. C.; Ruzza, C.; Malfacini, D.; Ferrari, F.; Bianco, S.; Costa, T.; Guerrini, R.; Trapella, C.; Calo', G. Pharmacological characterization of cebranopadol a novel analgesic acting as mixed nociceptin/orphanin FQ and opioid receptor agonist. *Pharmacology Research & Perspectives* **2016**, *4* (4), No. e00247.

(20) Cipriano, A.; Kapil, R. P.; Zhou, M.; Shet, M. S.; Whiteside, G. T.; Willsie, S. K.; Harris, S. C. Safety, Tolerability, and Pharmacokinetics of Single- and Multiple-Ascending Doses of Sunobinop in Healthy Participants. *Clinical pharmacology in drug development* **2024**, *13* (7), 790−800.

(21) Camilotto, R.; Malfacini, D.; Pola, P.; Morrone, E.; Frezza, A.; Ramalingam, R.; Sturaro, C.; Pacifico, S.; Ruzza, C.; Guerrini, R.; Whiteside, G.; Calo', G. In vitro pharmacological evaluation of the novel NOP receptor partial agonist sunobinop. *Biochemical pharmacology* **2025**, *238*, No. 116972.

(22) Camarda, V.; Fischetti, C.; Anzellotti, N.; Molinari, P.; Ambrosio, C.; Kostenis, E.; Regoli, D.; Trapella, C.; Guerrini, R.; Severo, S.; Calo, G. Pharmacological profile of NOP receptors coupled with calcium signaling via the chimeric protein G alpha qi5. *Naunyn-Schmiedeberg's archives of pharmacology* **2009**, *379* (6), 599−607.

(23) Malfacini, D.; Ambrosio, C.; Gro', M. C.; Sbraccia, M.; Trapella, C.; Guerrini, R.; Bonora, M.; Pinton, P.; Costa, T.; Calo', G. Pharmacological Profile of Nociceptin/Orphanin FQ Receptors Interacting with G-Proteins and β-Arrestins 2. *PLoS One* **2015**, *10* (8), No. e0132865.

(24) Calò, G.; Rizzi, A.; Bogoni, G.; Neugebauer, V.; Salvadori, S.; Guerrini, R.; Bianchi, C.; Regoli, D. The mouse vas deferens: a pharmacological preparation sensitive to nociceptin. *European journal of pharmacology* **1996**, *311* (1), R3−R5.

(25) Higgins, G. A.; Grottick, A. J.; Ballard, T. M.; Richards, J. G.; Messer, J.; Takeshima, H.; Pauly-Evers, M.; Jenck, F.; Adam, G.; Wichmann, J. Influence of the selective ORL1 receptor agonist, Ro64-6198, on rodent neurological function. *Neuropharmacology* **2001**, *41* (1), 97−107.

(26) Byford, A. J.; Anderson, A.; Jones, P. S.; Palin, R.; Houghton, A. K. The hypnotic, electroencephalographic, and antinociceptive properties of nonpeptide ORL1 receptor agonists after intravenous injection in rodents. *Anesthesia and analgesia* **2007**, *104* (1), 174−179.

(27) Pacifico, S.; Carotenuto, A.; Brancaccio, D.; Novellino, E.; Marzola, E.; Ferrari, F.; Cerlesi, M. C.; Trapella, C.; Preti, D.; Salvadori, S.; Calò, G.; Guerrini, R. Structure- and conformation-activity studies of nociceptin/orphanin FQ receptor dimeric ligands. *Sci. Rep.* **2017**, *7*, No. 45817.

(28) Busnelli, M.; Kleinau, G.; Muttenthaler, M.; Stoev, S.; Manning, M.; Bibic, L.; Howell, L. A.; McCormick, P. J.; Di Lascio, S.; Braida, D.; Sala, M.; Rovati, G. E.; Bellini, T.; Chini, B. Design and Characterization of Superpotent Bivalent Ligands Targeting Oxytocin Receptor Dimers via a Channel-Like Structure. *Journal of medicinal chemistry* **2016**, *59* (15), 7152−7166.

(29) Lensing, C. J.; Adank, D. N.; Wilber, S. L.; Freeman, K. T.; Schnell, S. M.; Speth, R. C.; Zarth, A. T.; Haskell-Luevano, C. A Direct in Vivo Comparison of the Melanocortin Monovalent Agonist Ac-His-DPhe-Arg-Trp-NH_2 versus the Bivalent Agonist Ac-His-

https://doi.org/10.1021/acs.jmedchem.5c02350
J. Med. Chem. XXXX, XXX, XXX−XXX

DPhe-Arg-Trp-PEDG20-His-DPhe-Arg-Trp-NH_2: A Bivalent Advantage. *ACS chemical neuroscience* **2017**, *8* (6), 1262−1278.
(30) Cowell, S. M.; Lee, Y. S. Biphalin: The Foundation of Bivalent Ligands. *Curr. Med. Chem.* **2016**, *23* (29), 3267−3284.
(31) Ferré, S.; Casadó, V.; Devi, L. A.; Filizola, M.; Jockers, R.; Lohse, M. J.; Milligan, G.; Pin, J. P.; Guitart, X. G protein-coupled receptor oligomerization revisited: functional and pharmacological perspectives. *Pharmacol. Rev.* **2014**, *66* (2), 413−434.
(32) Han, J.; Huang, Y.; Chen, X.; Zhou, F.; Fei, Y.; Fu, J. Rational design of dimeric lipidated Xenopus glucagon-like peptide 1 analogues as long-acting antihyperglycaemic agents. *European journal of medicinal chemistry* **2018**, *157*, 177−187.
(33) Pan, Y.; Shi, S.; Lao, X.; Zhang, J.; Tan, S.; Wu, Z.; Huang, J. A novel GLP-1 analog, a dimer of GLP-1 via covalent linkage by a lysine, prolongs the action of GLP-1 in the treatment of type 2 diabetes. *Peptides* **2017**, *88*, 46−54.
(34) Bracci, L.; Falciani, C.; Lelli, B.; Lozzi, L.; Runci, Y.; Pini, A.; De Montis, M. G.; Tagliamonte, A.; Neri, P. Synthetic peptides in the form of dendrimers become resistant to protease activity. *J. Biol. Chem.* **2003**, *278* (47), 46590−46595.
(35) Falciani, C.; Lozzi, L.; Pini, A.; Corti, F.; Fabbrini, M.; Bernini, A.; Lelli, B.; Niccolai, N.; Bracci, L. Molecular basis of branched peptides resistance to enzyme proteolysis. *Chemical biology & drug design* **2007**, *69* (3), 216−221.
(36) Bird, M. F.; Cerlesi, M. C.; Brown, M.; Malfacini, D.; Vezzi, V.; Molinari, P.; Micheli, L.; Di Cesare Mannelli, L.; Ghelardini, C.; Guerrini, R.; Calò, G.; Lambert, D. G. Characterisation of the Novel Mixed Mu-NOP Peptide Ligand Dermorphin-N/OFQ (DeNo). *PLoS One* **2016**, *11* (6), No. e0156897.
(37) Kawano, S.; Ito, R.; Nishiyama, M.; Kubo, M.; Matsushima, T.; Minamisawa, M.; Ambo, A.; Sasaki, Y. Receptor binding properties and antinociceptive effects of chimeric peptides consisting of a micro-opioid receptor agonist and an ORL1 receptor antagonist. *Biological & pharmaceutical bulletin* **2007**, *30* (7), 1260−1264.
(38) Guillemyn, K.; Starnowska, J.; Lagard, C.; Dyniewicz, J.; Rojewska, E.; Mika, J.; Chung, N. N.; Utard, V.; Kosson, P.; Lipkowski, A. W.; Chevillard, L.; Arranz-Gibert, P.; Teixidó, M.; Megarbane, B.; Tourwé, D.; Simonin, F.; Przewlocka, B.; Schiller, P. W.; Ballet, S. Bifunctional Peptide-Based Opioid Agonist-Nociceptin Antagonist Ligands for Dual Treatment of Acute and Neuropathic Pain. *Journal of medicinal chemistry* **2016**, *59* (8), 3777−3792.
(39) Calo', G.; Rizzi, A.; Ruzza, C.; Ferrari, F.; Pacifico, S.; Gavioli, E. C.; Salvadori, S.; Guerrini, R. Peptide welding technology - A simple strategy for generating innovative ligands for G protein coupled receptors. *Peptides* **2018**, *99*, 195−204.
(40) Pacifico, S.; Albanese, V.; Illuminati, D.; Fantinati, A.; Marzola, E.; Ferrari, F.; Neto, J. A.; Sturaro, C.; Ruzza, C.; Calò, G.; Preti, D.; Guerrini, R. Tetrabranched Hetero-Conjugated Peptides as Bifunctional Agonists of the NOP and Mu Opioid Receptors. *Bioconjugate Chem.* **2019**, *30* (9), 2444−2451.
(41) Rizzi, A.; Malfacini, D.; Cerlesi, M. C.; Ruzza, C.; Marzola, E.; Bird, M. F.; Rowbotham, D. J.; Salvadori, S.; Guerrini, R.; Lambert, D. G.; Calo, G. In vitro and in vivo pharmacological characterization of nociceptin/orphanin FQ tetrabranched derivatives. *British journal of pharmacology* **2014**, *171* (17), 4138−4153.
(42) Guerrini, R.; Marzola, E.; Trapella, C.; Pela', M.; Molinari, S.; Cerlesi, M. C.; Malfacini, D.; Rizzi, A.; Salvadori, S.; Calo', G. A novel and facile synthesis of tetra branched derivatives of nociceptin/orphanin FQ. *Bioorganic & medicinal chemistry* **2014**, *22* (14), 3703−3712.
(43) Rizzi, A.; Sukhtankar, D. D.; Ding, H.; Hayashida, K.; Ruzza, C.; Guerrini, R.; Calò, G.; Ko, M. C. Spinal antinociceptive effects of the novel NOP receptor agonist PWT2-nociceptin/orphanin FQ in mice and monkeys. *British journal of pharmacology* **2015**, *172* (14), 3661−3670.
(44) Kenakin, T. *A Pharmacology Primer: Techniques for More Effective and Strategic Drug Discovery*, 5th ed.; Academic Press: London, 2021.
(45) Reinscheid, R. K.; Ardati, A.; Monsma, F. J., Jr; Civelli, O. Structure-activity relationship studies on the novel neuropeptide orphanin FQ. *J. Biol. Chem.* **1996**, *271* (24), 14163−14168.
(46) Berzetei-Gurske, I. P.; Schwartz, R. W.; Toll, L. Determination of activity for nociceptin in the mouse vas deferens. *European journal of pharmacology* **1996**, *302* (1−3), R1−R2.
(47) Camarda, V.; Calo', G. Chimeric G proteins in fluorimetric calcium assays: experience with opioid receptors. *Methods in molecular biology* **2013**, *937*, 293−306.
(48) Kilkenny, C.; Browne, W. J.; Cuthill, I. C.; Emerson, M.; Altman, D. G. Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. *PLoS biology* **2010**, *8* (6), No. e1000412.
(49) Nishi, M.; Houtani, T.; Noda, Y.; Mamiya, T.; Sato, K.; Doi, T.; Kuno, J.; Takeshima, H.; Nukada, T.; Nabeshima, T.; Yamashita, T.; Noda, T.; Sugimoto, T. Unrestrained nociceptive response and disregulation of hearing ability in mice lacking the nociceptin/orphaninFQ receptor. *EMBO journal* **1997**, *16* (8), 1858−1864.
(50) Briscini, L.; Corradini, L.; Ongini, E.; Bertorelli, R. Up-regulation of ORL-1 receptors in spinal tissue of allodynic rats after sciatic nerve injury. *European journal of pharmacology* **2002**, *447* (1), 59−65.
(51) Holanda, V. A. D.; Pacifico, S.; Azevedo Neto, J.; Finetti, L.; Lobão-Soares, B.; Calo, G.; Gavioli, E. C.; Ruzza, C. Modulation of the NOP receptor signaling affects resilience to acute stress. *Journal of psychopharmacology (Oxford, England)* **2019**, *33* (12), 1540−1549.
(52) Rizzi, A.; Vergura, R.; Marzola, G.; Ruzza, C.; Guerrini, R.; Salvadori, S.; Regoli, D.; Calo, G. Neuropeptide S is a stimulatory anxiolytic agent: a behavioural study in mice. *British journal of pharmacology* **2008**, *154* (2), 471−479.
(53) Neubig, R. R.; Spedding, M.; Kenakin, T.; Christopoulos, A. International Union of Pharmacology Committee on Receptor Nomenclature and Drug Classification. XXXVIII. Update on terms and symbols in quantitative pharmacology. *Pharmacol. Rev.* **2003**, *55* (4), 597−606.
(54) Alexander, S. P. H.; Christopoulos, A.; Davenport, A. P.; Kelly, E.; Mathie, A. A.; Peters, J. A.; Veale, E. L.; Armstrong, J. F.; Faccenda, E.; Harding, S. D.; Davies, J. A.; Abbracchio, M. P.; Abraham, G.; Agoulnik, A.; Alexander, W.; Al-Hosaini, K.; Bäck, M.; Baker, J. G.; Barnes, N. M.; Bathgate, R.; Ye, R. D.; et al. The Concise Guide to PHARMACOLOGY 2023/24: G protein-coupled receptors. *British journal of pharmacology* **2023**, *180 Suppl. 2*, S23−S144.

https://doi.org/10.1021/acs.jmedchem.5c02350
J. Med. Chem. XXXX, XXX, XXX−XXX

GENERAL CONCLUSIONS

Migraine is one of the most prevalent and debilitating neurological disorders worldwide. In recent years, research has greatly advanced our understanding of its pathophysiology and led to the development of innovative and highly effective therapeutic strategies, primarily focused on the inhibition of CGRP signaling. However, several aspects still leave room for the discovery of novel pharmacological approaches. Although CGRP-targeting treatments are generally well tolerated and well established as preventive therapies, issues such as comorbidities, poor adherence, high costs, and uncertainties regarding long-term safety remain critical concerns [149]. For instance, in Italy, the I-GRAINE database revealed that preventive treatments targeting CGRP or its receptor are effective in 69-79% of the cases, leaving more than 20% of migraineurs seeking effective therapies [150]. For these reasons, the identification of new molecular targets for migraine therapy and prevention is timely and necessary. Within this context, our work identifies the NOP receptor as an actionable target whose peripheral activation is sufficient to blunt migraine-like pain.

The present work investigated the role of the endogenous N/OFQ and of the NOP receptor in preclinical models of migraine, using PMA as the primary readout. We employed two complementary paradigms: (i) an acute model in which a single administration of CGRP evokes PMA, and (ii) a stress-sensitized model in which restraint stress followed by a subthreshold dose of GTN reliably induces PMA. The latter paradigm more closely reflects the human condition, where stress is among the most frequent migraine triggers/facilitators, and, when tested in aged mice, yields persistent allodynia that models chronic progression. Notably, PMA did not differ between NOP wild-type and NOP knockout mice in either paradigm, indicating that endogenous N/OFQ signaling does not measurably contribute under these specific experimental conditions. Among the findings of this work, the most intriguing evidence comes from the pharmacological studies with NOP agonists. While the lack of difference between NOP wild-type and knockout mice indicates that endogenous N/OFQ release does not contribute to migraine-like pain under our experimental conditions, exogenous activation of the receptor revealed a clear therapeutic potential. In particular, peripherally restricted NOP agonism matched the efficacy of a brain-penetrant agonist in preventing / reversing PMA across all the experimental paradigms tested, providing robust evidence that the peripheral NOP receptor represents a therapeutically relevant site of action. At the cellular level, we

further demonstrated that NOP activation inhibits CGRP-induced cAMP production in human Schwann cells and recruits endosomal Gαi signaling, offering a plausible mechanism underlying the peripheral anti-migraine effects. However, this aspect requires further investigation. Only the use of conditional knockout models selectively lacking NOP receptors in Schwann cells will allow us to determine whether the antimigraine efficacy of NOP agonists truly depends on NOP activation in these cells or involves additional peripheral or central mechanisms. Nevertheless, this work paves the way for a new therapeutic paradigm, in which NOP receptor activation counteracts CGRP-driven mechanisms, not only in migraine but potentially also in other chronic pain conditions where CGRP plays a pathogenic role, such as neuropathic pain and endometriosis [151], [152].

The evidence that peripheral NOP receptor activation effectively prevents migraine-like pain highlights the therapeutic potential of peripherally restricted NOP agonists. Such compounds, which do not cross the blood–brain barrier (BBB), are particularly attractive for migraine therapy, as they would avoid the central side effects associated with NOP activation in the brain, the most significant of which is sedation [102], [153]. Moreover, it has been reported that central NOP activation may, under specific conditions, increase susceptibility to stress [122], [125]. Therefore, restricting NOP agonism to the periphery represents a rational and safer pharmacological strategy. A straightforward approach to achieve peripheral selectivity is the use of peptide-based molecules, which, due to their chemical and physical properties, are poorly BBB-permeable. Peptides also offer additional advantages as drug candidates: they display high target selectivity, minimizing off-target effects; they have a predictable metabolic profile and do not generate toxic metabolites. However, their development has been hampered by unfavorable pharmacokinetics, largely due to rapid enzymatic degradation by peptidases, which limits their half-life and in vivo efficacy. To address this limitation, we focused on improving peptide stability and duration of action through dimerization, a strategy that can enhance resistance to enzymatic cleavage while preserving receptor affinity and functional selectivity.

Thus, a series of novel dimeric N/OFQ-derived peptides was designed and screened for their potency and efficacy toward the NOP receptor. The best compound, 1h, resulted 3-fold more potent than N/OFQ in inducing the loss of the RR reflex in mice. Importantly, dimerization markedly prolonged its duration of

action, making it a promising candidate for further in vivo evaluation in migraine models.

Taken together, this work provides a comprehensive preclinical characterization of the NOP receptor as an innovative target for migraine therapy. The findings demonstrate that selective activation of peripheral NOP receptors effectively counteracts migraine-like pain, supporting its relevance as a novel target for therapeutic intervention. Moreover, the successful design of dimeric N/OFQ-based agonists highlights dimerization as a viable strategy to generate peripherally restricted, long-acting NOP agonists. Overall, these results pave the way for the development of a new generation of safe and effective anti-migraine agents, and potentially for broader applications in other CGRP-driven chronic pain conditions.

BIBLIOGRAPHY

[1] P. J. Goadsby and S. Evers, “International Classification of Headache Disorders - ICHD-4 *alpha*,” *Cephalalgia*, vol. 40, no. 9, pp. 887–888, Aug. 2020, doi: 10.1177/0333102420919098.

[2] L. Stovner *et al.*, “The global burden of headache: a documentation of headache prevalence and disability worldwide.,” *Cephalalgia*, vol. 27, no. 3, pp. 193–210, Mar. 2007, doi: 10.1111/j.1468-2982.2007.01288.x.

[3] R. Burch, P. Rizzoli, and E. Loder, “The prevalence and impact of migraine and severe headache in the United States: Updated age, sex, and socioeconomic-specific estimates from government health surveys.,” *Headache*, vol. 61, no. 1, pp. 60–68, Jan. 2021, doi: 10.1111/head.14024.

[4] T. W. Victor, X. Hu, J. C. Campbell, D. C. Buse, and R. B. Lipton, “Migraine prevalence by age and sex in the United States: a life-span study.,” *Cephalalgia*, vol. 30, no. 9, pp. 1065–1072, Sep. 2010, doi: 10.1177/0333102409355601.

[5] D. C. Buse *et al.*, “Impact of migraine on the family: perspectives of people with migraine and their spouse/domestic partner in the cameo study.,” *Mayo Clin. Proc.*, Apr. 2016, doi: 10.1016/j.mayocp.2016.02.013.

[6] P. J. Goadsby, R. B. Lipton, and M. D. Ferrari, “Migraine--current understanding and treatment.,” *N. Engl. J. Med.*, vol. 346, no. 4, pp. 257–270, Jan. 2002, doi: 10.1056/NEJMra010917.

[7] L. Kelman, “The triggers or precipitants of the acute migraine attack.,” *Cephalalgia*, vol. 27, no. 5, pp. 394–402, May 2007, doi: 10.1111/j.1468-2982.2007.01303.x.

[8] R. B. Lipton *et al.*, “Reduction in perceived stress as a migraine trigger: testing the ‘let-down headache’ hypothesis.,” *Neurology*, vol. 82, no. 16, pp. 1395–1401, Apr. 2014, doi: 10.1212/WNL.0000000000000332.

[9] C. Wacogne, J. P. Lacoste, E. Guillibert, F. C. Hugues, and C. Le Jeunne, “Stress, anxiety, depression and migraine.,” *Cephalalgia*, vol. 23, no. 6, pp. 451–455, Jul. 2003, doi: 10.1046/j.1468-2982.2003.00550.x.

[10] A. I. Scher, W. F. Stewart, D. Buse, D. S. Krantz, and R. B. Lipton, “Major life changes before and after the onset of chronic daily headache: a population-based study.,” *Cephalalgia*, vol. 28, no. 8, pp. 868–876, Aug. 2008, doi: 10.1111/j.1468-2982.2008.01634.x.

[11] H. Yapıcı-Eser, B. Dönmez-Demir, K. Kılıç, E. Eren-Koçak, and T. Dalkara, “Stress modulates cortical excitability via α-2 adrenergic and glucocorticoid receptors: As assessed by spreading depression.,” *Exp. Neurol.*, vol. 307, pp. 45–51, Sep. 2018, doi: 10.1016/j.expneurol.2018.05.024.

[12] M. Balkaya *et al.*, “Relief following chronic stress augments spreading depolarization susceptibility in familial hemiplegic migraine mice.,” *Neuroscience*, vol. 415, pp. 1–9, Sep. 2019, doi: 10.1016/j.neuroscience.2019.07.006.

[13] C. Robert *et al.*, “Paraventricular hypothalamic regulation of trigeminovascular mechanisms involved in headaches.,” *J. Neurosci.*, vol. 33, no. 20, pp. 8827–8840, May 2013, doi: 10.1523/JNEUROSCI.0439-13.2013.

[14] E. L. Spierings, M. Sorbi, G. H. Maassen, and P. C. Honkoop, “Psychophysical precedents of migraine in relation to the time of onset of the headache: the migraine time line.,” *Headache*, vol. 37, no. 4, pp. 217–220, Apr. 1997, doi: 10.1046/j.1526-4610.1997.3704217.x.

[15] T. A. Smitherman, D. B. Penzien, and M. Maizels, “Anxiety disorders and migraine intractability and progression.,” *Curr. Pain Headache Rep.*, vol. 12, no. 3, pp. 224–229, Jun. 2008, doi: 10.1007/s11916-008-0039-9.

[16] S. Ashina *et al.*, “Depression and risk of transformation of episodic to chronic migraine.,” *J. Headache Pain*, vol. 13, no. 8, pp. 615–624, Nov. 2012, doi: 10.1007/s10194-012-0479-9.

[17] P. J. Goadsby, P. R. Holland, M. Martins-Oliveira, J. Hoffmann, C. Schankin, and S. Akerman, “Pathophysiology of migraine: A disorder of sensory processing.,” *Physiol. Rev.*, vol. 97, no. 2, pp. 553–622, Apr. 2017, doi: 10.1152/physrev.00034.2015.

[18] L.-M. Terrier, N. Hadjikhani, and C. Destrieux, “The trigeminal pathways.,” *J. Neurol.*, vol. 269, no. 7, pp. 3443–3460, Jul. 2022, doi: 10.1007/s00415-022-11002-4.

[19] A. Ebersberger, B. Averbeck, K. Messlinger, and P. W. Reeh, “Release of substance P, calcitonin gene-related peptide and prostaglandin E2 from rat dura mater encephali following electrical and chemical stimulation in vitro.,” *Neuroscience*, vol. 89, no. 3, pp. 901–907, Mar. 1999, doi: 10.1016/s0306-4522(98)00366-2.

[20] R. Burstein *et al.*, “Thalamic sensitization transforms localized pain into widespread allodynia.,” *Ann. Neurol.*, vol. 68, no. 1, pp. 81–91, Jul. 2010, doi: 10.1002/ana.21994.

[21] R. Burstein, R. Noseda, and D. Borsook, “Migraine: multiple processes, complex pathophysiology.,” *J. Neurosci.*, vol. 35, no. 17, pp. 6619–6629, Apr. 2015, doi: 10.1523/JNEUROSCI.0373-15.2015.

[22] F. De Logu *et al.*, “Schwann cell endosome CGRP signals elicit periorbital mechanical allodynia in mice.,” *Nat. Commun.*, vol. 13, no. 1, p. 646, Feb. 2022, doi: 10.1038/s41467-022-28204-z.

[23] X. Zhang, D. Levy, R. Noseda, V. Kainz, M. Jakubowski, and R. Burstein, “Activation of meningeal nociceptors by cortical spreading depression: implications for migraine with aura.,” *J. Neurosci.*, vol. 30, no. 26, pp. 8807–8814, Jun. 2010, doi: 10.1523/JNEUROSCI.0511-10.2010.

[24] X. Zhang, D. Levy, V. Kainz, R. Noseda, M. Jakubowski, and R. Burstein, “Activation of central trigeminovascular neurons by cortical spreading depression.,” *Ann. Neurol.*, vol. 69, no. 5, pp. 855–865, May 2011, doi: 10.1002/ana.22329.

[25] E. Spekker, M. Tanaka, Á. Szabó, and L. Vécsei, “Neurogenic inflammation: the participant in migraine and recent advancements in translational research.,” *Biomedicines*, vol. 10, no. 1, Dec. 2021, doi: 10.3390/biomedicines10010076.

[26] H. Bolay, U. Reuter, A. K. Dunn, Z. Huang, D. A. Boas, and M. A. Moskowitz, “Intrinsic brain activity triggers trigeminal meningeal afferents in a migraine model.,” *Nat. Med.*, vol. 8, no. 2, pp. 136–142, Feb. 2002, doi: 10.1038/nm0202-136.

[27] L.-M. Terrier *et al.*, “The trigeminal system: The meningovascular complex- A review.,” *J. Anat.*, vol. 239, no. 1, pp. 1–11, Jul. 2021, doi: 10.1111/joa.13413.

[28] J. J. Y. Ong and M. De Felice, “Correction to: migraine treatment: current acute medications and their potential mechanisms of action.,” *Neurotherapeutics*, vol. 15, no. 2, pp. 525–526, Apr. 2018, doi: 10.1007/s13311-017-0599-7.

[29] S. D. Silberstein and S. H. Kori, “Dihydroergotamine: a review of formulation approaches for the acute treatment of migraine.,” *CNS Drugs*, vol. 27, no. 5, pp. 385–394, May 2013, doi: 10.1007/s40263-013-0061-2.

[30] J. C. Oswald and N. M. Schuster, “Lasmiditan for the treatment of acute migraine: a review and potential role in clinical practice.,” *J. Pain Res.*, vol. 11, pp. 2221–2227, Oct. 2018, doi: 10.2147/JPR.S152216.

[31] F. Zobdeh *et al.*, “Pharmacological treatment of migraine: Drug classes, mechanisms of action, clinical trials and new treatments.,” *Br. J. Pharmacol.*, vol. 178, no. 23, pp. 4588–4607, Dec. 2021, doi: 10.1111/bph.15657.

[32] A. Labastida-Ramírez *et al.*, “Lasmiditan inhibits calcitonin gene-related peptide release in the rodent trigeminovascular system.,” *Pain*, vol. 161, no. 5, pp. 1092–1099, May 2020, doi: 10.1097/j.pain.0000000000001801.

[33] D. W. Dodick *et al.*, “Ubrogepant for the treatment of migraine.,” *N. Engl. J. Med.*, vol. 381, no. 23, pp. 2230–2241, Dec. 2019, doi: 10.1056/NEJMoa1813049.

[34] T. P. Do, S. Guo, and M. Ashina, “Correction to: Therapeutic novelties in migraine: new drugs, new hope?,” *J. Headache Pain*, vol. 20, no. 1, p. 55, May 2019, doi: 10.1186/s10194-019-1013-0.

[35] A. R. Pavelic, C. Wöber, F. Riederer, and K. Zebenholzer, “Monoclonal Antibodies against Calcitonin Gene-Related Peptide for Migraine Prophylaxis: A Systematic Review of Real-World Data.,” *Cells*, vol. 12, no. 1, Dec. 2022, doi: 10.3390/cells12010143.

[36] “Correction: Melo-Carrillo et al., ‘Selective Inhibition of Trigeminovascular Neurons by Fremanezumab: A Humanized Monoclonal Anti-CGRP Antibody’.,” *J. Neurosci.*, vol. 37, no. 45, p. 11067, Nov. 2017, doi: 10.1523/JNEUROSCI.3012-17.2017.

[37] F. Cohen, H. Yuan, and S. D. Silberstein, “Calcitonin Gene-Related Peptide (CGRP)-Targeted Monoclonal Antibodies and Antagonists in Migraine: Current Evidence and Rationale.,” *BioDrugs*, vol. 36, no. 3, pp. 341–358, May 2022, doi: 10.1007/s40259-022-00530-0.

[38] S. Sacco *et al.*, “European Headache Federation guideline on the use of monoclonal antibodies targeting the calcitonin gene related peptide pathway for migraine prevention - 2022 update.,” *J. Headache Pain*, vol. 23, no. 1, p. 67, Jun. 2022, doi: 10.1186/s10194-022-01431-x.

[39] K. Drellia, L. Kokoti, C. I. Deligianni, D. Papadopoulos, and D. D. Mitsikostas, “Anti-CGRP monoclonal antibodies for migraine prevention: A systematic review and likelihood to help or harm analysis.,” *Cephalalgia*, vol. 41, no. 7, pp. 851–864, Jun. 2021, doi: 10.1177/0333102421989601.

[40] D. W. Dodick *et al.*, “Risk of hypertension in erenumab-treated patients with migraine: Analyses of clinical trial and postmarketing data.,” *Headache*, vol. 61, no. 9, pp. 1411–1420, Oct. 2021, doi: 10.1111/head.14208.

[41] P. Barbanti *et al.*, “Predictors of response to anti-CGRP monoclonal antibodies: a 24-week, multicenter, prospective study on 864 migraine patients.,” *J. Headache Pain*, vol. 23, no. 1, p. 138, Nov. 2022, doi: 10.1186/s10194-022-01498-6.

[42] G. G. Graham, K. F. Scott, and R. O. Day, “[Tolerability of paracetamol].,” *Drugs*, vol. 63 Spec No 2, pp. 43–46, 2003.

[43] H. C. Diener, V. Pfaffenrath, L. Pageler, H. Peil, and B. Aicher, “The fixed combination of acetylsalicylic acid, paracetamol and caffeine is more effective than single substances and dual combination for the treatment of headache: a multicentre, randomized, double-blind, single-dose, placebo-controlled parallel group study.,” *Cephalalgia*, vol. 25, no. 10, pp. 776–787, Oct. 2005, doi: 10.1111/j.1468-2982.2005.00948.x.

[44] R. Colás Chacartegui, R. Temprano González, C. Gómez Arruza, P. Muñoz Cacho, and J. Pascual Gómez, “[Abuse pattern of analgesics in chronic daily headache: a study in the general population].,” *Rev. Clin. Esp.*, vol. 205, no. 12, pp. 583–587, Dec. 2005, doi: 10.1016/s0014-2565(05)72650-3.

[45] C. J. Vierck, P. T. Hansson, and R. P. Yezierski, “Clinical and pre-clinical pain assessment: are we measuring the same thing?,” *Pain*, vol. 135, no. 1–2, pp. 7–10, Mar. 2008, doi: 10.1016/j.pain.2007.12.008.

[46] A. M. Harriott, L. C. Strother, M. Vila-Pueyo, and P. R. Holland, “Animal models of migraine and experimental techniques used to examine trigeminal sensory processing.,” *J. Headache Pain*, vol. 20, no. 1, p. 91, Aug. 2019, doi: 10.1186/s10194-019-1043-7.

[47] S. R. Chaplan, F. W. Bach, J. W. Pogrel, J. M. Chung, and T. L. Yaksh, “Quantitative assessment of tactile allodynia in the rat paw.,” *J. Neurosci. Methods*, vol. 53, no. 1, pp. 55–63, Jul. 1994, doi: 10.1016/0165-0270(94)90144-9.

[48] M. Ashina, J. M. Hansen, B. O. Á Dunga, and J. Olesen, “Human models of migraine - short-term pain for long-term gain.,” *Nat. Rev. Neurol.*, vol. 13, no. 12, pp. 713–724, Dec. 2017, doi: 10.1038/nrneurol.2017.137.

[49] M. Ashina, J. M. Hansen, and J. Olesen, “Pearls and pitfalls in human pharmacological models of migraine: 30 years’ experience.,” *Cephalalgia*, vol. 33, no. 8, pp. 540–553, Jun. 2013, doi: 10.1177/0333102412475234.

[50] W. Murrell, “NITRO-GLYCERINE AS A REMEDY FOR ANGINA PECTORIS.,” *Lancet*, vol. 113, no. 2890, pp. 80–81, Jan. 1879, doi: 10.1016/S0140-6736(02)46032-1.

[51] B. J. Rea *et al.*, “Peripherally administered calcitonin gene-related peptide induces spontaneous pain in mice: implications for migraine.,” *Pain*, vol. 159, no. 11, pp. 2306–2317, Nov. 2018, doi: 10.1097/j.pain.0000000000001337.

[52] F. De Logu *et al.*, “Migraine-provoking substances evoke periorbital allodynia in mice.,” *J. Headache Pain*, vol. 20, no. 1, p. 18, Feb. 2019, doi: 10.1186/s10194-019-0968-1.

[53] B. N. Mason *et al.*, “Induction of Migraine-Like Photophobic Behavior in Mice by Both Peripheral and Central CGRP Mechanisms.,” *J. Neurosci.*, vol. 37, no. 1, pp. 204–216, Jan. 2017, doi: 10.1523/JNEUROSCI.2967-16.2016.

[54] A. Costa, A. Smeraldi, C. Tassorelli, R. Greco, and G. Nappi, “Effects of acute and chronic restraint stress on nitroglycerin-induced hyperalgesia in rats.,” *Neurosci. Lett.*, vol. 383, no. 1–2, pp. 7–11, Jul. 2005, doi: 10.1016/j.neulet.2005.03.026.

[55] D. Kaufmann and K. C. Brennan, “The effects of chronic stress on migraine relevant phenotypes in male mice.,” *Front. Cell. Neurosci.*, vol. 12, p. 294, Sep. 2018, doi: 10.3389/fncel.2018.00294.

[56] M. Raoof *et al.*, “The effects of neonatal maternal deprivation and chronic unpredictable stresses on migraine-like behaviors in adult rats.,” *Neurosci. Lett.*, vol. 772, p. 136444, Feb. 2022, doi: 10.1016/j.neulet.2022.136444.

[57] A. Avona *et al.*, “Repetitive stress in mice causes migraine-like behaviors and calcitonin gene-related peptide-dependent hyperalgesic priming to a migraine trigger.,” *Pain*, vol. 161, no. 11, pp. 2539–2550, Nov. 2020, doi: 10.1097/j.pain.0000000000001953.

[58] C. M. Kopruszinski, E. Navratilova, J. Swiokla, D. W. Dodick, I. P. Chessell, and F. Porreca, “A novel, injury-free rodent model of vulnerability for assessment of acute and preventive therapies reveals temporal contributions of CGRP-receptor activation in migraine-like pain.,” *Cephalalgia*, vol. 41, no. 3, pp. 305–317, Mar. 2021, doi: 10.1177/0333102420959794.

[59] C. Mollereau *et al.*, “ORL1, a novel member of the opioid receptor family. Cloning, functional expression and localization.,” *FEBS Lett.*, vol. 341, no. 1, pp. 33–38, Mar. 1994, doi: 10.1016/0014-5793(94)80235-1.

[60] O. Civelli, R. K. Reinscheid, Y. Zhang, Z. Wang, R. Fredriksson, and H. B. Schiöth, “G protein-coupled receptor deorphanizations.,” *Annu. Rev. Pharmacol. Toxicol.*, vol. 53, pp. 127–146, 2013, doi: 10.1146/annurev-pharmtox-010611-134548.

[61] J. C. Meunier *et al.*, “Isolation and structure of the endogenous agonist of opioid receptor-like ORL1 receptor.,” *Nature*, vol. 377, no. 6549, pp. 532–535, Oct. 1995, doi: 10.1038/377532a0.

[62] R. K. Reinscheid *et al.*, “Orphanin FQ: a neuropeptide that activates an opioidlike G protein-coupled receptor.,” *Science*, vol. 270, no. 5237, pp. 792–794, Nov. 1995, doi: 10.1126/science.270.5237.792.

[63] B. M. Cox, M. J. Christie, L. Devi, L. Toll, and J. R. Traynor, “Challenges for opioid receptor nomenclature: IUPHAR Review 9.,” *Br. J. Pharmacol.*, vol. 172, no. 2, pp. 317–323, Jan. 2015, doi: 10.1111/bph.12612.

[64] D. G. Lambert, “The nociceptin/orphanin FQ receptor: a target with broad therapeutic potential.,” *Nat. Rev. Drug Discov.*, vol. 7, no. 8, pp. 694–710, Aug. 2008, doi: 10.1038/nrd2572.

[65] L. Toll, M. R. Bruchas, G. Calo’, B. M. Cox, and N. T. Zaveri, “Nociceptin/Orphanin FQ Receptor Structure, Signaling, Ligands, Functions, and Interactions with Opioid Systems.,” *Pharmacol. Rev.*, vol. 68, no. 2, pp. 419–457, Apr. 2016, doi: 10.1124/pr.114.009209.

[66] C. W. Stevens, “The evolution of vertebrate opioid receptors.,” *Front Biosci (Landmark Ed)*, vol. 14, pp. 1247–1269, Jan. 2009, doi: 10.2741/3306.

[67] A. A. Thompson *et al.*, “Structure of the nociceptin/orphanin FQ receptor in complex with a peptide mimetic.,” *Nature*, vol. 485, no. 7398, pp. 395–399, May 2012, doi: 10.1038/nature11085.

[68] F. Meng *et al.*, “Creating a functional opioid alkaloid binding site in the orphanin FQ receptor through site-directed mutagenesis.,” *Mol. Pharmacol.*, vol. 53, no. 4, pp. 772–777, Apr. 1998, doi: 10.1124/mol.53.4.772.

[69] P. R. Daga and N. T. Zaveri, “Homology modeling and molecular dynamics simulations of the active state of the nociceptin receptor reveal new insights into agonist binding and activation.,” *Proteins*, vol. 80, no. 8, pp. 1948–1961, Aug. 2012, doi: 10.1002/prot.24077.

[70] A. Ciancetta *et al.*, “A Multi-Angle Approach to Predict Peptide-GPCR Complexes: The N/OFQ-NOP System as a Successful AlphaFold Application Case Study.,” *J. Chem. Inf. Model.*, vol. 64, no. 20, pp. 8034–8051, Oct. 2024, doi: 10.1021/acs.jcim.4c00499.

[71] C. R. Neal *et al.*, “Opioid receptor-like (ORL1) receptor distribution in the rat central nervous system: comparison of ORL1 receptor mRNA expression with (125)I-[(14)Tyr]-orphanin FQ binding.,” *J. Comp. Neurol.*, vol. 412, no. 4, pp. 563–605, Oct. 1999, doi: 10.1002/(SICI)1096-9861(19991004)412:4<563::AID-CNE2>3.0.CO;2-Z.

[72] S. Florin, J. Meunier, and J. Costentin, “Autoradiographic localization of [3H]nociceptin binding sites in the rat brain.,” *Brain Res.*, vol. 880, no. 1–2, pp. 11–16, Oct. 2000, doi: 10.1016/s0006-8993(00)02669-x.

[73] C. R. Neal, A. Mansour, R. Reinscheid, H. P. Nothacker, O. Civelli, and S. J. Watson, “Localization of orphanin FQ (nociceptin) peptide and messenger RNA in the central nervous system of the rat.,” *J. Comp. Neurol.*, vol. 406, no. 4, pp. 503–547, Apr. 1999, doi: 10.1002/(SICI)1096-9861(19990419)406:4<503::AID-CNE7>3.0.CO;2-P.

[74] A. Ozawa *et al.*, “Knock-In Mice with NOP-eGFP Receptors Identify Receptor Cellular and Regional Localization.,” *J. Neurosci.*, vol. 35, no. 33, pp. 11682–11693, Aug. 2015, doi: 10.1523/JNEUROSCI.5122-14.2015.

[75] A. Ozawa *et al.*, “Analysis of the distribution of spinal NOP receptors in a chronic pain model using NOP-eGFP knock-in mice.,” *Br. J. Pharmacol.*, vol. 175, no. 13, pp. 2662–2675, Jul. 2018, doi: 10.1111/bph.14225.

[76] Y. Odagaki *et al.*, “Receptor-mediated Gi-3 activation in mammalian and human brain membranes: Reestablishment method and its application to nociceptin/orphanin FQ opioid peptide (NOP) receptor/Gi-3 interaction.,” *J. Pharmacol. Sci.*, vol. 158, no. 2, pp. 131–138, Jun. 2025, doi: 10.1016/j.jphs.2025.03.014.

[77] M. Connor, A. Yeo, and G. Henderson, “The effect of nociceptin on Ca2+ channel current and intracellular Ca2+ in the SH-SY5Y human neuroblastoma cell line.,” *Br. J. Pharmacol.*, vol. 118, no. 2, pp. 205–207, May 1996, doi: 10.1111/j.1476-5381.1996.tb15387.x.

[78] N. R. Zhang *et al.*, “Serine 363 is required for nociceptin/orphanin FQ opioid receptor (NOPR) desensitization, internalization, and arrestin signaling.,” *J. Biol. Chem.*, vol. 287, no. 50, pp. 42019–42030, Dec. 2012, doi: 10.1074/jbc.M112.405696.

[79] W. Armstead, “NOC/oFQ activates PKC and generates superoxide to impair hypotensive cerebrovasodilation after hypoxia/ischemia.,” *Med. Sci. Monit.*, vol. 8, no. 1, pp. BR8–BR14, Jan. 2002.

[80] Z. Zhang, S. M. Xin, G. X. Wu, W. B. Zhang, L. Ma, and G. Pei, “Endogenous delta-opioid and ORL1 receptors couple to phosphorylation and activation of p38 MAPK in NG108-15 cells and this is regulated by protein kinase A and protein kinase C.,” *J. Neurochem.*, vol. 73, no. 4, pp. 1502–1509, Oct. 1999, doi: 10.1046/j.1471-4159.1999.0731502.x.

[81] D. R. Thakker and K. M. Standifer, “Induction of G protein-coupled receptor kinases 2 and 3 contributes to the cross-talk between mu and ORL1 receptors following prolonged agonist exposure.,” *Neuropharmacology*, vol. 43, no. 6, pp. 979–990, Nov. 2002, doi: 10.1016/s0028-3908(02)00145-4.

[82] C. L. Donica, H. O. Awwad, D. R. Thakker, and K. M. Standifer, “Cellular mechanisms of nociceptin/orphanin FQ (N/OFQ) peptide (NOP) receptor regulation and heterologous regulation by N/OFQ.,” *Mol. Pharmacol.*, vol. 83, no. 5, pp. 907–918, May 2013, doi: 10.1124/mol.112.084632.

[83] C. Mustazza, S. Pieretti, and F. Marzoli, “Nociceptin /Orphanin FQ Peptide (NOP) Receptor Modulators: An Update in Structure-Activity Relationships.,” *Curr. Med. Chem.*, vol. 25, no. 20, pp. 2353–2384, 2018, doi: 10.2174/0929867325666180111095458.

[84] D. Preti, G. Caló, and R. Guerrini, “NOP-Targeted Peptide Ligands.,” *Handb. Exp. Pharmacol.*, vol. 254, pp. 17–36, 2019, doi: 10.1007/164_2018_198.

[85] J. L. Montiel, F. Cornille, B. P. Roques, and F. Noble, “Nociceptin/orphanin FQ metabolism: role of aminopeptidase and endopeptidase 24.15.,” *J. Neurochem.*, vol. 68, no. 1, pp. 354–361, Jan. 1997, doi: 10.1046/j.1471-4159.1997.68010354.x.

[86] C. Sakurada, S. Sakurada, T. Orito, K. Tan-No, and T. Sakurada, “Degradation of nociceptin (orphanin FQ) by mouse spinal cord synaptic membranes is triggered by endopeptidase-24.11: an in vitro and in vivo study.,” *Biochem. Pharmacol.*, vol. 64, no. 8, pp. 1293–1303, Oct. 2002, doi: 10.1016/s0006-2952(02)01295-9.

[87] F. Noble and B. P. Roques, “Association of aminopeptidase N and endopeptidase 24.15 inhibitors potentiate behavioral effects mediated by nociceptin/orphanin FQ in mice.,” *FEBS Lett.*, vol. 401, no. 2–3, pp. 227–229, Jan. 1997, doi: 10.1016/s0014-5793(96)01476-7.

[88] N. Kiguchi, H. Ding, and M.-C. Ko, “Central N/OFQ-NOP Receptor System in Pain Modulation.,” *Adv. Pharmacol.*, vol. 75, pp. 217–243, 2016, doi: 10.1016/bs.apha.2015.10.001.

[89] W. Schröder, D. G. Lambert, M. C. Ko, and T. Koch, “Functional plasticity of the N/OFQ-NOP receptor system determines analgesic properties of NOP receptor agonists.,” *Br. J. Pharmacol.*, vol. 171, no. 16, pp. 3777–3800, Aug. 2014, doi: 10.1111/bph.12744.

[90] L. Toll, A. Ozawa, and A. Cippitelli, “NOP-Related Mechanisms in Pain and Analgesia.,” *Handb. Exp. Pharmacol.*, vol. 254, pp. 165–186, 2019, doi: 10.1007/164_2019_214.

[91] G. Calò *et al.*, “Pharmacological characterization of the nociceptin receptor mediating hyperalgesia in the mouse tail withdrawal assay.,” *Br. J. Pharmacol.*, vol. 125, no. 2, pp. 373–378, Sep. 1998, doi: 10.1038/sj.bjp.0702087.

[92] Y. Q. Wang, C. B. Zhu, X. D. Cao, and G. C. Wu, “Supraspinal hyperalgesia and spinal analgesia by [Phe1psi(CH2-NH)Gly2]nociceptin-(1-13)-NH2 in rat.,” *Eur. J. Pharmacol.*, vol. 376, no. 3, pp. R1-3, Jul. 1999, doi: 10.1016/s0014-2999(99)00399-4.

[93] A. Rizzi *et al.*, “In vitro and in vivo studies on UFP-112, a novel potent and long lasting agonist selective for the nociceptin/orphanin FQ receptor.,” *Peptides*, vol. 28, no. 6, pp. 1240–1251, Jun. 2007, doi: 10.1016/j.peptides.2007.04.020.

[94] T. Yamamoto, N. Nozaki-Taguchi, and S. Kimura, “Analgesic effect of intrathecally administered nociceptin, an opioid receptor-like1 receptor agonist, in the rat formalin test.,” *Neuroscience*, vol. 81, no. 1, pp. 249–254, Nov. 1997, doi: 10.1016/s0306-4522(97)00166-8.

[95] M. A. King, G. C. Rossi, A. H. Chang, L. Williams, and G. W. Pasternak, “Spinal analgesic activity of orphanin FQ/nociceptin and its fragments.,” *Neurosci. Lett.*, vol. 223, no. 2, pp. 113–116, Feb. 1997, doi: 10.1016/s0304-3940(97)13414-0.

[96] T. Yamamoto, N. Nozaki-Taguchi, and S. Kimura, “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.*, vol. 754, no. 1–2, pp. 329–332, Apr. 1997, doi: 10.1016/s0006-8993(97)00186-8.

[97] C. Courteix, M.-A. Coudoré-Civiale, A.-M. Privat, T. Pélissier, A. Eschalier, and J. Fialip, “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*, vol. 110, no. 1–2, pp. 236–245, Jul. 2004, doi: 10.1016/j.pain.2004.03.037.

[98] Y. Chen and C. Sommer, “Activation of the nociceptin opioid system in rat sensory neurons produces antinociceptive effects in inflammatory pain: involvement of inflammatory mediators.,” *J. Neurosci. Res.*, vol. 85, no. 7, pp. 1478–1488, May 2007, doi: 10.1002/jnr.21272.

[99] F. M. Dautzenberg *et al.*, “Pharmacological characterization of the novel nonpeptide orphanin FQ/nociceptin receptor agonist Ro 64-6198: rapid and reversible desensitization of

the ORL1 receptor in vitro and lack of tolerance in vivo.," *J. Pharmacol. Exp. Ther.*, vol. 298, no. 2, pp. 812–819, Aug. 2001.

[100] F. Jenck *et al.*, "A synthetic agonist at the orphanin FQ/nociceptin receptor ORL1: anxiolytic profile in the rat.," *Proc Natl Acad Sci USA*, vol. 97, no. 9, pp. 4938–4943, Apr. 2000, doi: 10.1073/pnas.090514397.

[101] A. Rizzi *et al.*, "Pharmacological characterization of cebranopadol a novel analgesic acting as mixed nociceptin/orphanin FQ and opioid receptor agonist.," *Pharmacol. Res. Perspect.*, vol. 4, no. 4, p. e00247, Aug. 2016, doi: 10.1002/prp2.247.

[102] J. Azevedo Neto *et al.*, "Functional selectivity does not predict antinociceptive/locomotor impairing potencies of NOP receptor agonists.," *Front. Neurosci.*, vol. 15, p. 657153, Mar. 2021, doi: 10.3389/fnins.2021.657153.

[103] J. Kotlinska, J. Wichmann, P. Rafalski, S. Talarek, T. Dylag, and J. Silberring, "Non-peptidergic OP4 receptor agonist inhibits morphine antinociception but does not influence morphine dependence.," *Neuroreport*, vol. 14, no. 4, pp. 601–604, Mar. 2003, doi: 10.1097/00001756-200303240-00015.

[104] M.-C. Ko, J. H. Woods, W. E. Fantegrossi, C. M. Galuska, J. Wichmann, and E. P. Prinssen, "Behavioral effects of a synthetic agonist selective for nociceptin/orphanin FQ peptide receptors in monkeys.," *Neuropsychopharmacology*, vol. 34, no. 9, pp. 2088–2096, Aug. 2009, doi: 10.1038/npp.2009.33.

[105] A. P. Lin and M.-C. Ko, "The therapeutic potential of nociceptin/orphanin FQ receptor agonists as analgesics without abuse liability.," *ACS Chem. Neurosci.*, vol. 4, no. 2, pp. 214–224, Feb. 2013, doi: 10.1021/cn300124f.

[106] U. B. Depner, R. K. Reinscheid, H. Takeshima, K. Brune, and H. U. Zeilhofer, "Normal sensitivity to acute pain, but increased inflammatory hyperalgesia in mice lacking the nociceptin precursor polypeptide or the nociceptin receptor.," *Eur. J. Neurosci.*, vol. 17, no. 11, pp. 2381–2387, Jun. 2003, doi: 10.1046/j.1460-9568.2003.02676.x.

[107] A. Rizzi, S. Molinari, M. Marti, G. Marzola, and G. Calo', "Nociceptin/orphanin FQ receptor knockout rats: in vitro and in vivo studies.," *Neuropharmacology*, vol. 60, no. 4, pp. 572–579, Mar. 2011, doi: 10.1016/j.neuropharm.2010.12.010.

[108] C. Mollereau and L. Mouledous, "Tissue distribution of the opioid receptor-like (ORL1) receptor.," *Peptides*, vol. 21, no. 7, pp. 907–917, Jul. 2000, doi: 10.1016/s0196-9781(00)00227-8.

[109] F. Jenck *et al.*, "Orphanin FQ acts as an anxiolytic to attenuate behavioral responses to stress.," *Proc Natl Acad Sci USA*, vol. 94, no. 26, pp. 14854–14858, Dec. 1997, doi: 10.1073/pnas.94.26.14854.

[110] J. Wichmann, G. Adam, S. Röver, A. M. Cesura, F. M. Dautzenberg, and F. Jenck, "8-acenaphthen-1-yl-1-phenyl-1,3,8-triaza-spiro[4.5]decan-4-one derivatives as orphanin FQ receptor agonists.," *Bioorg. Med. Chem. Lett.*, vol. 9, no. 16, pp. 2343–2348, Aug. 1999, doi: 10.1016/s0960-894x(99)00385-6.

[111] G. B. Varty *et al.*, "Characterization of the nociceptin receptor (ORL-1) agonist, Ro64-6198, in tests of anxiety across multiple species.," *Psychopharmacology (Berl)*, vol. 182, no. 1, pp. 132–143, Oct. 2005, doi: 10.1007/s00213-005-0041-4.

[112] G. B. Varty *et al.*, "The anxiolytic-like effects of the novel, orally active nociceptin opioid receptor agonist 8-[bis(2-methylphenyl)methyl]-3-phenyl-8-azabicyclo[3.2.1]octan-3-ol (SCH 221510).," *J. Pharmacol. Exp. Ther.*, vol. 326, no. 2, pp. 672–682, Aug. 2008, doi: 10.1124/jpet.108.136937.

[113] A. Köster *et al.*, "Targeted disruption of the orphanin FQ/nociceptin gene increases stress susceptibility and impairs stress adaptation in mice.," *Proc Natl Acad Sci USA*, vol. 96, no. 18, pp. 10444–10449, Aug. 1999, doi: 10.1073/pnas.96.18.10444.

[114] J. P. Redrobe, G. Calo', D. Regoli, and R. Quirion, "Nociceptin receptor antagonists display antidepressant-like properties in the mouse forced swimming test.," *Naunyn Schmiedebergs Arch Pharmacol*, vol. 365, no. 2, pp. 164–167, Feb. 2002, doi: 10.1007/s00210-001-0511-0.

[115] E. C. Gavioli *et al.*, “Blockade of nociceptin/orphanin FQ-NOP receptor signalling produces antidepressant-like effects: pharmacological and genetic evidences from the mouse forced swimming test.,” *Eur. J. Neurosci.*, vol. 17, no. 9, pp. 1987–1990, May 2003, doi: 10.1046/j.1460-9568.2003.02603.x.

[116] E. C. Gavioli *et al.*, “Antidepressant-like effects of the nociceptin/orphanin FQ receptor antagonist UFP-101: new evidence from rats and mice.,” *Naunyn Schmiedebergs Arch Pharmacol*, vol. 369, no. 6, pp. 547–553, Jun. 2004, doi: 10.1007/s00210-004-0939-0.

[117] V. A. D. Holanda, I. U. Medeiros, L. Asth, R. Guerrini, G. Calo’, and E. C. Gavioli, “Antidepressant activity of nociceptin/orphanin FQ receptor antagonists in the mouse learned helplessness.,” *Psychopharmacology (Berl)*, vol. 233, no. 13, pp. 2525–2532, Jul. 2016, doi: 10.1007/s00213-016-4310-1.

[118] A. Rizzi *et al.*, “Pharmacological characterization of the nociceptin/orphanin FQ receptor antagonist SB-612111 [(-)-cis-1-methyl-7-[[4-(2,6-dichlorophenyl)piperidin-1-yl]methyl]-6,7,8,9-tetrahydro-5H-benzocyclohepten-5-ol]: in vivo studies.,” *J. Pharmacol. Exp. Ther.*, vol. 321, no. 3, pp. 968–974, Jun. 2007, doi: 10.1124/jpet.106.116780.

[119] F. D’Oliveira da Silva *et al.*, “The NOP antagonist BTRX-246040 increases stress resilience in mice without affecting adult neurogenesis in the hippocampus.,” *Neuropharmacology*, vol. 212, p. 109077, Jul. 2022, doi: 10.1016/j.neuropharm.2022.109077.

[120] G. Vitale *et al.*, “Chronic treatment with the selective NOP receptor antagonist [Nphe 1, Arg 14, Lys 15]N/OFQ-NH 2 (UFP-101) reverses the behavioural and biochemical effects of unpredictable chronic mild stress in rats.,” *Psychopharmacology (Berl)*, vol. 207, no. 2, pp. 173–189, Dec. 2009, doi: 10.1007/s00213-009-1646-9.

[121] A. Post *et al.*, “A Selective Nociceptin Receptor Antagonist to Treat Depression: Evidence from Preclinical and Clinical Studies.,” *Neuropsychopharmacology*, vol. 41, no. 7, pp. 1803–1812, Jun. 2016, doi: 10.1038/npp.2015.348.

[122] V. A. D. Holanda *et al.*, “Activation of NOP receptor increases vulnerability to stress: role of glucocorticoids and CRF signaling.,” *Psychopharmacology (Berl)*, vol. 241, no. 5, pp. 1001–1010, May 2024, doi: 10.1007/s00213-024-06533-9.

[123] A. B. Câmara and I. A. Brandão, “Behavioral and neurochemical effects of nociceptin/orphanin FQ receptor activation in the social defeat protocol.,” *Behav. Neurosci.*, vol. 137, no. 1, pp. 52–66, Feb. 2023, doi: 10.1037/bne0000539.

[124] E. C. Gavioli, V. A. D. Holanda, G. Calo, and C. Ruzza, “Nociceptin/orphanin FQ receptor system blockade as an innovative strategy for increasing resilience to stress.,” *Peptides*, vol. 141, p. 170548, Jul. 2021, doi: 10.1016/j.peptides.2021.170548.

[125] V. A. D. Holanda *et al.*, “Modulation of the NOP receptor signaling affects resilience to acute stress.,” *J Psychopharmacol (Oxford)*, vol. 33, no. 12, pp. 1540–1549, Dec. 2019, doi: 10.1177/0269881119864942.

[126] V. A. D. Holanda, M. C. Oliveira, E. D. Da Silva Junior, G. Calo’, C. Ruzza, and E. C. Gavioli, “Blockade of nociceptin/orphanin FQ signaling facilitates an active copying strategy due to acute and repeated stressful stimuli in mice.,” *Neurobiol. Stress*, vol. 13, p. 100255, Nov. 2020, doi: 10.1016/j.ynstr.2020.100255.

[127] P. Pola, A. Frezza, E. C. Gavioli, G. Calò, and C. Ruzza, “Effects of Stress Exposure to Pain Perception in Pre-Clinical Studies: Focus on the Nociceptin/Orphanin FQ-NOP Receptor System.,” *Brain Sci.*, vol. 14, no. 9, Sep. 2024, doi: 10.3390/brainsci14090936.

[128] J. Sandin, J. Georgieva, P. A. Schött, S. O. Ogren, and L. Terenius, “Nociceptin/orphanin FQ microinjected into hippocampus impairs spatial learning in rats.,” *Eur. J. Neurosci.*, vol. 9, no. 1, pp. 194–197, Jan. 1997, doi: 10.1111/j.1460-9568.1997.tb01367.x.

[129] T. Manabe *et al.*, “Facilitation of long-term potentiation and memory in mice lacking nociceptin receptors.,” *Nature*, vol. 394, no. 6693, pp. 577–581, Aug. 1998, doi: 10.1038/29073.

[130] R. Andero, “Nociceptin and the nociceptin receptor in learning and memory.,” *Prog Neuropsychopharmacol Biol Psychiatry*, vol. 62, pp. 45–50, Oct. 2015, doi: 10.1016/j.pnpbp.2015.02.007.

[131] F. D'Oliveira da Silva *et al.*, "Targeting Nociceptin/Orphanin FQ receptor to rescue cognitive symptoms in a mouse neuroendocrine model of chronic stress.," *Mol. Psychiatry*, vol. 29, no. 3, pp. 718–729, Mar. 2024, doi: 10.1038/s41380-023-02363-x.

[132] S. R. Morairty, Y. Sun, L. Toll, M. R. Bruchas, and T. S. Kilduff, "Activation of the nociceptin/orphanin-FQ receptor promotes NREM sleep and EEG slow wave activity.," *Proc Natl Acad Sci USA*, vol. 120, no. 13, p. e2214171120, Mar. 2023, doi: 10.1073/pnas.2214171120.

[133] G. A. Higgins *et al.*, "Influence of the selective ORL1 receptor agonist, Ro64-6198, on rodent neurological function.," *Neuropharmacology*, vol. 41, no. 1, pp. 97–107, Jul. 2001, doi: 10.1016/s0028-3908(01)00048-x.

[134] M. Nishi *et al.*, "Unrestrained nociceptive response and disregulation of hearing ability in mice lacking the nociceptin/orphaninFQ receptor.," *EMBO J.*, vol. 16, no. 8, pp. 1858–1864, Apr. 1997, doi: 10.1093/emboj/16.8.1858.

[135] A. J. Byford, A. Anderson, P. S. Jones, R. Palin, and A. K. Houghton, "The hypnotic, electroencephalographic, and antinociceptive properties of nonpeptide ORL1 receptor agonists after intravenous injection in rodents.," *Anesth. Analg.*, vol. 104, no. 1, pp. 174–179, Jan. 2007, doi: 10.1213/01.ane.0000250403.88649.51.

[136] G. T. Whiteside *et al.*, "The nociceptin/orphanin FQ receptor partial agonist sunobinop promotes non-REM sleep in rodents and patients with insomnia.," *J. Clin. Invest.*, vol. 134, no. 1, Jan. 2024, doi: 10.1172/JCI171172.

[137] G. X. Xie, T. Meuser, C. Pietruck, M. Sharma, and P. P. Palmer, "Presence of opioid receptor-like (ORL1) receptor mRNA splice variants in peripheral sensory and sympathetic neuronal ganglia.," *Life Sci.*, vol. 64, no. 22, pp. 2029–2037, 1999, doi: 10.1016/s0024-3205(99)00150-2.

[138] K. M. Targowska-Duda *et al.*, "NOP receptor agonist attenuates nitroglycerin-induced migraine-like symptoms in mice.," *Neuropharmacology*, vol. 170, p. 108029, Jun. 2020, doi: 10.1016/j.neuropharm.2020.108029.

[139] M. Hou, R. Uddman, J. Tajti, and L. Edvinsson, "Nociceptin immunoreactivity and receptor mRNA in the human trigeminal ganglion.," *Brain Res.*, vol. 964, no. 2, pp. 179–186, Feb. 2003, doi: 10.1016/s0006-8993(02)03927-6.

[140] J. Witta, M. Palkovits, J. Rosenberger, and B. M. Cox, "Distribution of nociceptin/orphanin FQ in adult human brain.," *Brain Res.*, vol. 997, no. 1, pp. 24–29, Jan. 2004, doi: 10.1016/j.brainres.2003.08.066.

[141] S. L. Borgland, M. Connor, and M. J. Christie, "Nociceptin inhibits calcium channel currents in a subpopulation of small nociceptive trigeminal ganglion neurons in mouse.," *J Physiol (Lond)*, vol. 536, no. Pt 1, pp. 35–47, Oct. 2001, doi: 10.1111/j.1469-7793.2001.t01-1-00035.x.

[142] A. Capuano *et al.*, "Nociceptin (1-13)NH2 inhibits stimulated calcitonin-gene-related-peptide release from primary cultures of rat trigeminal ganglia neurones.," *Cephalalgia*, vol. 27, no. 8, pp. 868–876, Aug. 2007, doi: 10.1111/j.1468-2982.2007.01354.x.

[143] A. Capuano, A. De Corato, G. Tringali, D. Currò, C. Dello Russo, and P. Navarra, "Buprenorphine inhibits bradykinin-induced release of calcitonin gene-related peptide from rat trigeminal neurons via both mu-opioid and nociceptin/orphanin peptide receptors.," *Eur. J. Pharmacol.*, vol. 609, no. 1–3, pp. 45–50, May 2009, doi: 10.1016/j.ejphar.2009.03.013.

[144] C. A. Flores, X. M. Wang, K. M. Zhang, and S. S. Mokha, "Orphanin FQ produces gender-specific modulation of trigeminal nociception: behavioral and electrophysiological observations.," *Neuroscience*, vol. 105, no. 2, pp. 489–498, 2001, doi: 10.1016/s0306-4522(01)00179-8.

[145] X. M. Wang, K. M. Zhang, and S. S. Mokha, "Nociceptin (orphanin FQ), an endogenous ligand for the QRL1 (opioid-receptor-like1) receptor; modulates responses of trigeminal neurons evoked by excitatory amino acids and somatosensory stimuli.," *J. Neurophysiol.*, vol. 76, no. 5, pp. 3568–3572, Nov. 1996, doi: 10.1152/jn.1996.76.5.3568.

[146] T. Bartsch, S. Akerman, and P. J. Goadsby, "The ORL-1 (NOP1) receptor ligand nociceptin/orphanin FQ (N/OFQ) inhibits neurogenic dural vasodilatation in the rat.,"

Neuropharmacology, vol. 43, no. 6, pp. 991–998, Nov. 2002, doi: 10.1016/s0028-3908(02)00148-x.

[147] A. Mudgal *et al.*, “Ro 64-6198, a selective NOP receptor agonist attenuates social impairments associated with NTG-induced migraine pain.,” *Neuropsychopharmacology*, Aug. 2025, doi: 10.1038/s41386-025-02187-z.

[148] C. Ertsey, M. Hantos, G. Bozsik, and K. Tekes, “Plasma nociceptin levels are reduced in migraine without aura.,” *Cephalalgia*, vol. 25, no. 4, pp. 261–266, Apr. 2005, doi: 10.1111/j.1468-2982.2004.00849.x.

[149] A. C. Charles, K. B. Digre, P. J. Goadsby, M. S. Robbins, A. Hershey, and American Headache Society, “Calcitonin gene-related peptide-targeting therapies are a first-line option for the prevention of migraine: An American Headache Society position statement update.,” *Headache*, vol. 64, no. 4, pp. 333–341, Apr. 2024, doi: 10.1111/head.14692.

[150] P. Barbanti *et al.*, “The first report of the Italian Migraine Registry (I-GRAINE).,” *Neurol. Sci.*, vol. 43, no. 9, pp. 5725–5728, Sep. 2022, doi: 10.1007/s10072-022-06214-5.

[151] V. Fattori *et al.*, “Nonsurgical mouse model of endometriosis-associated pain that responds to clinically active drugs.,” *Pain*, vol. 161, no. 6, pp. 1321–1331, Jun. 2020, doi: 10.1097/j.pain.0000000000001832.

[152] S. Iyengar, M. H. Ossipov, and K. W. Johnson, “The role of calcitonin gene-related peptide in peripheral and central pain mechanisms including migraine.,” *Pain*, vol. 158, no. 4, pp. 543–559, Apr. 2017, doi: 10.1097/j.pain.0000000000000831.

[153] B. E. Pierce *et al.*, “NOP agonist AT-403 promoted sleep in lactic acid-induced acute pain model.,” *Front Pain Res (Lausanne)*, vol. 6, p. 1659121, Sep. 2025, doi: 10.3389/fpain.2025.1659121.