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New target for the development of antimigraine drugs: in vitro and in vivo study.

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ABSTRACT

Migraine is a recurrent headache disorder typified by painful attacks lasting 4 to 72 hours, which affects 12% of the Caucasian population and has a major impact on the well-being and quality of life of patients and their families. As a multifactorial disorder, the pathophysiology of migraine is very complex and can be diverse among patients. The trigeminovascular theory, which includes changes in the vasculature, central and peripheral pain processing, and inflammation is currently the most accredited to describe the disorder. Migraine can be treated by several different drug classes, divided into two macro-categories: symptomatic and preventive therapy. These treatments include serotoninergic agonists, drugs targeting the CGRP system, anticonvulsants, beta-blockers, tricyclic antidepressants, and others. Of note, most of these therapies are poorly tolerated or ineffective, a large number of chronic migraine patients are dissatisfied with their treatment, and some patients develop chronic migraine upon abuse of acute therapies. Hence the need to identify new therapeutic targets for the development of new anti-migraine drugs. The general aim of this research work has been the *in vitro* or the *in vivo* pharmacological investigation of new and standard compounds acting on different receptors involved in migraine disease.

The specific aims of the study were:

1. the *in vitro* set-up and pharmacological validation of a battery of assays for the pharmacological characterization of kappa opioid receptor ligands;
2. the design, synthesis, and pharmacological characterization of mixed NOP/opioid peptide agonists;
3. the design, synthesis, and pharmacological characterization of new TRPA1 antagonists, analogs of the standard antagonist DHC200;
4. the evaluation of cannabidiol (CBD) in vivo, in a mouse model of migraine induced by calcitonin gene-related peptide (CGRP) systemic administration;
5. the evaluation of the role played by the nociceptin/orphanin FQ receptor (NOP) in migraine by studying the phenotype of mice knockout for the NOP receptor (NOP(-/-)) in two experimental migraine models (nitroglycerin (GTN)-induced migraine and CGRP-induced migraine).

The main results can be resumed as follow:

1. a platform of *in vitro* assays to characterize ligands for the kappa opioid receptors have been successfully set up and pharmacologically validated using a panel of standard kappa ligands. These assays include calcium mobilization in cells expressing chimeric G proteins, the DMR label-free assay, and a BRET assay that allows measurement of receptor interaction with G

protein and β-arrestin 2. In the frame of this study, two new dynorphins A derivatives have been characterized: PWT2-Dyn A and Dyn A-palmitic. These compounds behaved as potent full kappa agonists;

2. 31 new compounds with the general sequence [Tyr/Dmt^{1}Xaa^{5}]N/OFQ(1-13)NH_2 have been synthesized and investigated through the calcium mobilization and the DMR assays, using cells stably expressing the NOP, mu, delta, and kappa receptors. [Dmt^{1,5}]N/OFQ(1-13)-NH_2 was identified as the most potent mixed NOP/mu peptide agonist so far described. This is a promising peptide to test in *in vivo* in pre-clinical migraine models;
3. DHC236 and DHC277 were identified as pure and potent TRPA1 antagonists, three times more potent than the starting compound DHC200. These are promising ligands to test in *in vivo* in pre-clinical migraine models;
4. a single administration of CGRP induced facial hypersensitivity in both female and male mice, while repeated CGRP treatment produced progressively decreased levels in basal pain thresholds only in female mice, suggesting the progression to a chronic migraine phase. In the acute protocol, the CBD administration protected both female and male mice from periorbital allodynia induced by a single CGRP injection, and in the chronic one prevented increased levels of basal allodynia induced by repeated CGRP treatment in female mice. Moreover, CBD injected after CGRP, reversed CGRP-evoked allodynia, and also reduced spontaneous pain traits induced by CGRP administration in female mice. Finally, CBD blocked CGRP-induced anxiety in male mice but failed in protecting CGRP-induced photophobia in female mice.
5. female and male NOP(-/-) mice were more sensitive to the effects of both GTN and CGRP compared to wild-type mice, suggesting that the NOP receptor plays a role in migraine onset.

In conclusion, this work brings to the scientific community new methodologies, compounds, and evidence useful to speed up the identification and development of new anti-migraine drugs.

Table of content

Abbreviation list

(2-propylpentanoic acid) VPA
4-hydroxynoneal (4-HNE)
Abdominal migraine (AM)
Adenosine triphosphate (ATP)
American Migraine Prevalence and Preventions (AMPP)
Benign approximal vertigo (BPV)
Benign paroxysmal torticollis (BPT)
Blood-brain barrier (BBB)
Calcitonin gene releated peptide (CGRP)
Calcitonin receptor-like receptor (CLR)
Cannabidiol (CBD)
Central nervous system (CNS)
Central nervous system (CNS)
Chronic cluster headache (CCH)
Chronic TTH (CTTH)
Cortical Spreading Depression (CSD)
Corticotropin-releasing factor (CRF)
Cyclic vomiting syndrome (CVS)
Cycloxygenase 1 (COX1)
Cycloxygenase 2 (COX2)
Delta-opioid receptor (DOR
Dhyidroergotamine (DHE)
Disability-adjusted life-years (DALYs)
Dorsal root ganglion (DRG)
Dorsal trigemino-thalamic tracts (DTT)
Episodic cluster headache (ECH)
Episodic TTH (ETTH)
Familial hemiplegic migraine (FHM)
Functional gastrointestinal disorders (FGIDs)
Global Burden of Disease (GBD)
Glycerin trinitrate (GTN)
Interferon γ (IFN-γ)
Interleukin 10 (IL-10)
Interleukin 6 (IL-&9
Kappa opioid receptro (KOR)
Lipopolysaccaride (LPS)
Magnetic resonance angiography (MRA)
Medication-overuse headaches (MOH)
Mesencephalic trigeminal nucleus (MN)
Middle cerebral artery (MCA)
Middle meningeal artery (MMA)
Monoclonal antibodies for CGRP (mAbs)
Mu-opioid receptor (MOR)

N-methyl-D-aspartate (NMDA)
NADPH oxidase 1 (NOX1)
NADPH oxidase 2 (NOX2)
Nitric oxide (NO)
NOD-like receptor protein 3 (NLRP3)
Nonsteroidal anti-inflammatory drugs (NSAIDs)
Nuclear factor-kappa B (NF-kB)
Paclitaxel (PAC)
Pituitary adenylate cyclase-activating polypeptide (PACAP)
Principal trigmenial nucleus (PN)
Proliferator-activated receptor gamma (PPARγ)
Prostaglandin E2 (PGE2)
Radical oxygen species (ROS)
Radical oxygen species (ROS)
Reactive carbonyl species (RCS)
Reactive nitrogen species (RNS)
Receptor activity-modifying protein 1 (RAMP1)
Spinal trigeminal Nucleus (SN)
Spinal trigeminal Nucleus pars caudalis (SNc)
Spinal trigeminal Nucleus pars interpolaris (SNi)
Spinal trigeminal Nucleus pars oralis (SNo)
Substance P (SP)
Tension type headache (TTH)
Transient receptor potential cation channel superfamily (TRP)
Transient receptor potential cation channel, subfamily A, member 1 (TRPA1)
Trigeminal autonomic cephalalgias (TACs)
Trigeminal cervical complex (TCC)
Trigeminal ganglia (TG)
Trigeminal nucleus caudalis (TNC)
Trigeminovascular system (TGVS)
TRP ankyrin (TRPA)
TRP canonical (TRPC)
TRP melastatin (TRPM)
TRP mucolipin (TRPML)
TRP polycystin (TRPP)
TRP vanilloid (TRPV)
Tumor necrosis factor (TNF)
Vagal ganglia (VG)
Vasoactive intestinal peptide (VIP)
Ventral trigemino-thalamic tracts (VTT)
Ventro-posterolateral (VPL)
Ventro-posteromedial (VPM)
Years of life lived with disability (YLDs)
Years of life lost (YLLs)

α-ammino 3-hydroxy 5-methyl 4-isoxazolpropionic (AMPA)
Δ9-tetrahydrocannabidinol (Δ9-THC)

1. INTRODUCTION

1.1 General definition and classification of headaches

According to the International Headache Society (ICHD-3 2018), headaches can be primary, if they are not attributable to a specific cause, or secondary, if they are a symptom of another disease. Secondary headaches may be due to several dysfunctions such as trauma or injury to the head and/or neck, cranial and/or cervical vascular disorder, to a substance or its withdrawal, caused by disorders of the skull, eyes, neck, ears, sinuses, teeth, nose, mouth or other facial or cervical structures or can be attributed to infections. Primary headaches can be divided into i) migraine, ii) tension-type headaches (TTH), and iii) trigeminal autonomic cephalagias (TACs). All these headache types are outlined in Table 1.

Table 1. Primary headaches type

Type	Location	Type of pain	Duration	Symptoms
Migraine	Unilateral	Pulsating	4 – 72 hours can become chronic	● Nausea or vomiting, ● photophobia, ● phonophobia ● cutaneous allodynia
Tension-type headache (TTH)	Bilateral	pressing or tightening (non-pulsating) quality	30 minutes - 7 days	● no nausea or vomiting ● no more than one of photophobia or phonophobia
TACs	unilateral orbital, supra- orbital and/or temporal pain	Orbital, supraorbital, and/or temporal	15 – 180 minutes	● at least one of the following symptoms or signs, ipsilateral to the headache: i. conjunctival injection and/or lacrimation ii. nasal congestion and/or rhinorrhea iii. eyelid oedema iv. forehead and facial sweating v. miosis and/or ptosis ● a sense of restlessness or agitation

Information in the table taken from (ICHD-3 2018)

Migraine – is a primary headache disorder and could be divided into two major types: i) migraine without aura and ii) migraine with aura (R B Lipton et al. 2004). Migraine without aura, previously called ‘common migraine’, is a pervasive but poorly understood nervous

system disorder characterized by long-lasting (4-72h) intense, pulsating pain often accompanied by light sensitivity (photophobia), nausea, vomiting, aggravation by physical activity, and comorbid anxiety and depression (ICHD-3 2018). Migraine with aura is characterized by fully reversible sensory, visual, or other neurological symptoms that often precede migraine onset, but sometimes accompany the headache (Peters 2019). In some patients, migraine can be preceded (from a few hours to 48 hours) or followed by a series of symptoms including hyperactivity, hypoactivity, depression, fatigue, difficulty with concentration, and neck pain, called a prodromal phase and postdromal phase, both in migraine with and without aura (ICHD-3 2018).

In the migraine without aura in children and adolescents, the pain is more often bilateral than in the case of adults; for them, migraine headache is unilateral and usually frontotemporal (ICHD-3 2018). Migraine attacks can be associated with symptoms of cutaneous allodynia. In some women, migraine attacks, predominantly without aura, are associated with their menstrual cycles. These kinds of attacks tend to be longer and accompanied by more severe nausea than attacks outside the menstrual cycle and they are classified as a per se migraine type in the ICHD-3 (Kjersti G Vetvik et al. 2014; Kjersti Grøtta Vetvik et al. 2015). Very frequent migraine attacks are distinguished as *chronic migraine.* The ICHD-3 defined a migraine chronic when it occurs 15 or more days/month for more than 3 months, and when associated with medication overuse are classified as *medication-overuse headaches (MOH)*; these types of migraine are the most common cause of symptoms suggestive of chronic migraine. During attacks of migraine without aura, there are no changes in blood flow that are typical in Cortical Spreading Depression (CSD) although some blood flow changes may occur as cortical changes secondary to pain activation (Charles and Brennan 2010).

Many patients who have migraine attacks without aura may also experience attacks with aura. The aura is a complex spectrum of neurological symptoms. Visual aura is the most common type (occurring in over 90% of patients) and appears as a zigzag figure near the point of fixation that may move to the left or right. It may start as a small hole of light, sometimes bright geometrical lines and shapes in the visual field. This visual aura may expand into a sickle- or C-shaped object, with zigzag lines on the leading edge. As it moves, it may appear to grow. Auras are not the same for all people, so some people also might experience bright spots or flashes. Auras are sometimes accompanied by a partial loss of vision referred to as a *scotoma*. This phenomenon commonly lasts 30 minutes (He, Li, and Nie 2015). Another typical neurological symptom is sensory disturbances. In this case, it begins as a tingling in one limb or a feeling of numbness moving slowly from the point of origin and affecting a part of the body, over 10 to 20 minutes. The sensation can spread to

one side of the face and tongue. Last in terms of frequency is speech disturbances referred to as dysphasic aura. When aura symptoms are multiple, they follow one another in succession, beginning with the visual one and ending with the speech disturbance (K. Jensen et al. 1986). In the rarest of auras, the limbs and possibly the face on one side of the body might become weak; this is referred to as *hemiplegic migraine* or one of its subforms. F*amilial hemiplegic migraine* (FHM) is a rare genetic form of hemiplegic migraine: *i)* in FHM1 there is a mutation in the CACNA1A gene (coding for a calcium channel) on chromosome 19; *ii)* in FHM2 the gene involved is the ATP1A2 (coding for a potassium/sodium-ATPase) on chromosome 1; *iii)* FHM3 that showed a mutation in the SCN1A gene (coding for a sodium channel) on chromosome 2. Sometimes, the FHM can be mistaken for epilepsy and treated as such (de Vries et al. 2009).

There are also a group of disorders in patients affected by migraine, called in the International Classification of Headache Disorders (ICHD-III, 2018), *episodic syndromes that may be associated with migraine*. It defines clinical entities that usually begin in childhood, but may also occur in adults. Some authors believe that could be a common genetic background between some episodic syndromes and FHM (Cuenca-León et al. 2008). Five episodic syndromes are currently recognized: *i)* cyclic vomiting syndrome (CVS); *ii)* abdominal migraine (AM); *iii)* benign approximal vertigo (BPV); *iv)* benign paroxysmal torticollis (BPT); *v)* infantile colic. The CVS, AM, and infantile colic involving the gastrointestinal tract are also recognized as functional gastrointestinal disorders (Hyams et al. 2016). The CVS is characterized by periodic episodes of nausea and vomiting divided into four phases: premonitory; emetic; recovery, and inter-episodic well phase. The duration of each phase is variable, in the case of the inter-episodic well phase the duration could be about several weeks or months. CVS often occurs around 5 years, with a predictable periodicity, but sometimes attacks could trigger by stress, and less often by menstruation. More rarely CVS occurs in adults, with a mean age of around 25 years (Gelfand 2015). In the case of AM the pediatric population affected by this pathology is around 4%, and it manifests as recurrent attacks of abdominal pain, lasting from 2 to 72 hours. These episodes are associated with anorexia, nausea/vomiting, or pallor. It usually shows up in school-aged children and disappears before 18, and the affected children often developed migraine in adulthood (Kunishi et al. 2016). The BPV appears at the age of 2/5 years until 6 years and is characterized by recurrent attacks of vertigo resolving spontaneously with a duration of a few minutes to hours (Gelfand 2015). Episodes are associated with nystagmus, ataxia, vomiting, and pallor, and children are often fearful during the attacks. Sometimes these attacks can persist into adolescence and more rarely the beginning of adulthood (Krams et

al. 2011). On the contrary, BPT is considered to occur only in infancy and is characterized by periodic and stereotyped attacks of cervical dystonia lasting from hours to days. Occurs typically around six months and resolves within three years. Episodes are associated whit vomiting, ataxia, pallor, and migrainous features (i.e. photophobia, phonophobia, vomiting, and motor sensitivity) that could be developed later in childhood (Krams et al. 2011; Greene et al. 2021). Finally, infantile colic is a frequent disorder that affects 1 baby in five. It manifests like episodes of irritability and crying in a healthy and well-fed baby. Some authors suggest that infantile colic indicates a state of increased allodynia, as is observed in children with migraine. This association with migraine is supported by a prospective study that shows that infants with colic had a three-fold risk to develop migraine without aura in adolescence. There is also an association between parental migraine and infantile colic (Abu-Arafeh and Gelfand 2021; Sillanpää and Saarinen 2015; Gelfand 2015).

Tension-type headache (TTH) – called also *muscle contraction headache* or *stress headache* in the ICHD-III 2018, is a non-migraine headache, generally characterized by mild to moderate pain that's often described as feeling like a tight band around the head. Is an oppressive and constant headache on both sides of the head, which tends to radiate to the neck and shoulders. This kind of headache is very common, with a prevalence in the general population of 79%. Even if it is the most common kind of headache, it gets much less attention from health authorities, clinical researchers, or industrial pharmacologists than migraine does, owing to the fact that most people with TTH do not consult a doctor but treat themselves, if necessary, with over-the-counter analgesics (Fumal and Schoenen 2008). It is also characterized by the absence of common migraine symptoms, such as photophobia, phonophobia, and nausea (Sait Ashina et al. 2021). TTH is divided into three subtypes: *i)* infrequent episodic TTH (ETTH, headache for less than 1 day per month); *ii)* frequent ETTH (headaches for 14 days per month); *iii)* chronic TTH (CTTH, headaches for more than 15 days per months). All these three subtypes are typically bilateral, pressing, or tightening in quality and of mild to moderate intensity, lasting minutes to days, in the case of infrequent and frequent ETTH, while in the CTTH the attacks could last hours to days, or unremitting (ICHD-3 2018).

Cluster headache – it is classified in the ICHD-3 under the *trigeminal autonomic cephalalgias (TACs).* TACs are characterized by unilateral trigeminal distribution pain that occurs in association with prominent ipsilateral cranial autonomic features (P J Goadsby and Lipton 1997). All the TACs types are outlined in Table 2. Cluster headache is characterized

by attacks of severe, strictly unilateral pain accompanied by ipsilateral conjunctival injection, watery eyes, nasal congestion, rhinorrhea, sweating of the forehead and face, miosis, ptosis, and eyelid edema, and restlessness or agitation (ICHD-3 2018). As the name suggests, attacks occur in series lasting for weeks or months, separated by remission periods. This kind of headache could be divided into two types: *i)* episodic cluster headache, and *ii)* chronic cluster headache. The attacks could be provoked by alcohol, histamine, or nitroglycerin. The pain is orbitally and supraorbitally, and during the attack, the patient is unable to lie down. Age at onset is around 20-40 years, and men are afflicted three times more often than women.

Table 2. TACs headaches.

Type	Location	Type of pain	Duration	Symptoms
Cluster headache	unilateral orbital, supra- orbital and/or temporal pain	Severe or very severe	15–180 minutes	• at least one of the following symptoms or signs, ipsilateral to the headache: i. conjunctival injection and/or lacrimation ii. nasal congestion and/or rhinorrhea iii. eyelid oedema iv. forehead and facial sweating v. miosis and/or ptosis • a sense of restlessness or agitation
Paroxysmal hemicrania	Unilateral orbital, supraorbital and/or temporal pain	Severe	2 – 30 minutes (many times a day)	• at least one of the following symptoms or signs, ipsilateral to the headache: i. conjunctival injections and/or lacrimation ii. nasal congestion and/or rhinorrhoea iii. eyelid oedema iv. forehead and facial sweating v. miosis and/or ptosis • occurring whit a frequency of >5 per day
Short-lasting unilateral neuralgiform headache attacks	Unilateral head pain with orbital, supraorbital temporal and/or other trigeminal distribution	Moderate or severe	Lasting seconds to minutes at least one a day	• at least one of the following symptoms or signs, ipsilateral to the headache: i. Conjunctival injection and/or lacrimation ii. Nasal congestion and/or rhinorrhoea iii. eyelid oedema iv. forehead and facial sweating v. miosis and/or ptosis
Hemicrania continua	Strictly unilateral headache	Chronic and persistent	Persistent	• at least one of the following symptoms or signs, ipsilateral to the headache: i. Conjunctival injection and/or lacrimation ii. Nasal congestion and/or rhinorrhoea iii. eyelid oedema iv. forehead and facial sweating v. miosis and/or ptosis • a sense of restlessness or agitation, or aggravation of the pain by movement

Information in the table taken from (ICHD-3 2018).

Other primary headache disorders – there are a number of primary headaches that are clinically heterogeneous and coded in under this heading in the ICHD-3. They are grouped into four categories:

i) Headaches associated with physical exertion are acknowledged:
   a. Primary cough headache
   b. Primary exercise headache
   c. Primary headache associated with sexual activity
   d. Primary thunderclap headache

ii) Headaches attributed to direct physical stimuli (non-damaging stimuli) such as:
   a. Cold-stimulus headache
   b. External pressure headache

iii) Epicranial headaches, including:
   a. Primary stabbing headache
   b. Nummular headache

iv) Other miscellaneous primary headache disorders include:
   a. Hypnic headache
   b. New daily persistent headache

The pathogenesis of these disorders is not understood. Headaches with similar characteristics to those above can be secondary headaches. For a more specific classification see (ICHD-3 2018).

Secondary headaches are a consequence of another disorder capable of causing it. A wide range of different conditions is accepted to be causal to headaches and includes head or neck trauma, vascular disorders, intracranial neoplasms, facial or cranial structure disorder, acute substance use or its withdrawal, and intracranial infection. Moreover, a secondary headache can only be definitively diagnosed if the headache is resolved after the cause has been eliminated. In real life, however, such a causal relationship cannot always be established and the headache can become chronic even when the underlying cause is resolved (e.g. post-traumatic headache after minor head trauma). In the ICHD-3 under the classification of *Headaches attributed to a substance or its withdrawal* (group 8) are classified some types of secondary headaches, including i) Nitric oxide (NO) donor-induced headache, ii) Medication-overuse headache (MOH). Secondary headaches can occur after a substance's withdrawal, such as caffeine withdrawal, opioid-withdrawal, or estrogen withdrawal.

NO donor-induced headache – it is well-known that glycerin trinitrate (GTN) causes typical headaches, which is a side effect of its therapeutic use. Other NO donors used for this purpose may also produce headaches such as isosorbide mononitrate which causes longer-lasting headaches than GTN, owing to its slow release of NO (Ingelise Christiansen et al. 2008). Several pieces of evidence are available in the literature. For instance, in a double-blind, placebo-controlled study performed in migraineurs, a delayed headache occurred after GTN-infusion in eight out of ten patients (J Olesen, Thomsen, and Iversen 1994; I Christiansen et al. 1999). Besides head pain, the GTN-induced migraine is characterized by sensitivity to light and sound, and by additional central nervous system symptoms, including neck stiffness, concentration problems, and irritability, even including premonitory symptoms (Giffin et al. 2003; Afridi, Kaube, and Goadsby 2004).

Medication-overuse headache (MOH) – is called also "drug-induced headache" and is a direct consequence of regular overuse of symptomatic headache medication for more than three months. Epidemiologically, more than half of people with headaches on 15 (or more) days per month have MOH and its prevalence is 1% in the general population (Linde et al. 2011; Westergaard et al. 2014; Russell and Lundqvist 2012). MOH can be due to the abuse of opioids, triptans and ergotamine for more than 9 days per month, or acetaminophen and nonsteroidal anti-inflammatory drugs (NSAIDs) for more than 14 days in a month (Peters 2019). This condition is an interaction between an overused drug and a sensitive patient. MOH patient has a very low quality of life and works productivity, moreover the presence of comorbidities, especially psychiatric, and sleep disturbance results in high costs and compromised social relationships (Linde et al. 2011; Westergaard et al. 2014). This particular type of secondary headache is really difficult to treat and the mechanism by which it develops is not fully understood.

1.2 Epidemiology of headache disorders

Headache is an almost universal symptom for which the causes are complex and heterogeneous. Indeed, half of the world's adult population has an active headache disorder (Stovner et al. 2007). This chapter will describe the epidemiology of many primary headache disorders. We first consider migraine, followed by TTH headache, cluster headache, and then MOH headache.

Epidemiology of migraine – Migraine without aura affects more than 10% of the world's population (Stovner et al. 2007), and has a strong impact on the individual and society: it is

the third most frequent disease and the second most debilitating for the mankind according to the World Health Organization. Several epidemiological studies have been conducted to assess the incidence and prevalence of this pathology. In 1-year prevalence study, conducted for the Eurolight project, data from nine European countries have been collected. This study reported a prevalence of 35% people (Steiner et al. 2014), in contrast with the prevalence reported for the US countries: 12-13% (R B Lipton et al. 2007). This difference could be due to differences in methodology and in the study population, indeed, the Eurolight project considered both definite and probable migraine, while the US project only includes definite migraine. If we consider that the probable migraine in the European study accounted for about 40% of cases, the discrepancy in the data was thus explained (M. Ashina, Katsarava, et al. 2021). According to the 2016 Lancet Global Burden of Disease (GBD) study, the prevalence of migraine across geographical regions is highest in Italy and Nepal and lowest in China.

In GBD, disease burden is estimated in disability-adjusted life-years (DALYs), which are the sum of years of life lost (YLLs) to premature mortality and years of life lived with disability (YLDs). YLDs for each headache disorder are calculated based on their prevalence and the average time patients spend with this type of headache multiplied by the weight of the associated disability. Migraine was estimated to have caused 45·1 million YLDs in 2016 (GBD 2016 Headache Collaborators 2018).

Migraine prevalence is age-related and sex-related (Figure 1), and is significantly more common in women (Buse et al. 2013).

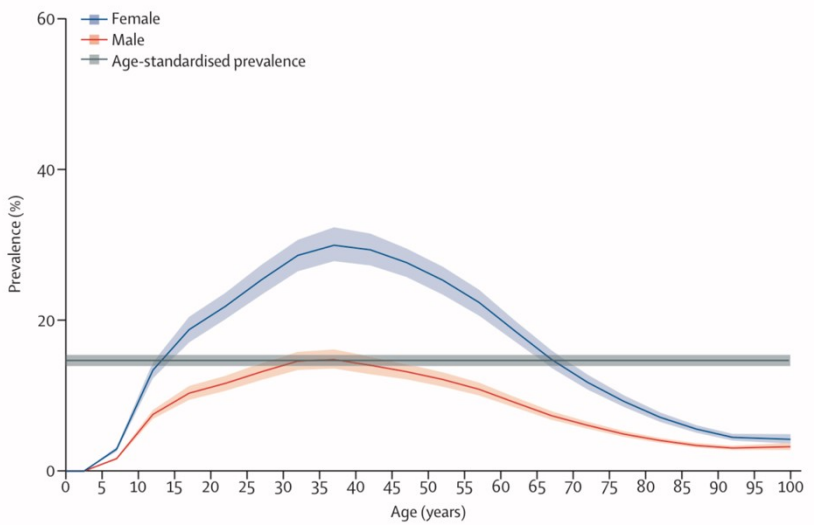

Figure 1. world prevalence of migraine normalized by age and gender. Figure taken from Global Burden of Disease Study 2016 (GBD 2016 Headache Collaborators 2018).

Its prevalence peaks in those aged 35 -39 years (GBD 2016 Headache Collaborators 2018), with a relatively lower prevalence in children, adolescents, and older people. In women

between ages 15 and 49, migraines caused 20·3 million YLDs in 2016. In another study, it was demonstrated that the woman-to-man prevalence ratio varies with age. The American Migraine Prevalence and Preventions (AMPP) study shows a higher prevalence in boys than in girls before puberty. This prevalence gradually increases rapidly in women after puberty, with a peak at age 17 (Buse et al. 2013; M E Bigal et al. 2007).

Migraine is not only more common in women than in men but is also more painful and severe. Women with migraine, in comparison to men, report higher pain intensity, headache-related disability, and more associated symptoms (Richard B Lipton et al. 2013). This difference may be due to hormonal factors, indeed 50% of menstrual cycles occur in 7.6% of all women and in 22% of women with migraine (Kjersti G Vetvik et al. 2014). Additionally, there are studies that indicate that the brain shows functional and structural differences between men and women with migraine, compared with healthy controls, during the reproductive years (Kjersti Grøtta Vetvik and MacGregor 2017). The fluctuating concentrations of female sex hormones across the menstrual cycle also have an effect on migraine characteristics, and the migraine attacks are longer, and more often associated with nausea when compared with non-menstrual attacks (Kjersti Grøtta Vetvik et al. 2015; Pinkerman and Holroyd 2010). As said a few pages before, also the so-called "estrogen-withdrawal" is associated with a higher prevalence of migraine which occurs premenstrually and during the hormone-free interval of the hormonal pill. This withdrawal could directly trigger migraine, or lower the attack threshold (E Anne MacGregor 2013; E A MacGregor et al. 2006). To support this, a study conducted in 2016 shows how women with migraine have a faster late luteal estrogen decline when compared with healthy controls, indicating a neuroendocrine vulnerability in these women (Pavlović et al. 2016).

Even in migraine-animal models, it's evident a similar trend. In some studies, female rodents show higher nociceptive responses to different stimuli than males (Araya et al. 2020; Alarcón-Alarcón et al. 2022). Again, this sexual dimorphism is demonstrated also in (Viero et al. 2022), where unpredictable sound stress causes higher nociceptive and anxiety-like behavior in females than in males.

In both men and women, migraine could be accompanied by comorbid with other diseases such as epilepsy, chronic pain syndromes, allergies, asthma, circulatory disorders, and psychiatric disorders (Scher, Bigal, and Lipton 2005; R. Jensen and Stovner 2008; Tietjen et al. 2007). Data from a Danish study shows that women with migraine had more comorbidity than men (11 vs 5), with a high incidence of thyroid diseases and musculoskeletal symptoms (Le et al. 2011). On the contrary, a Norwegian study did not find any significant sex difference in musculoskeletal symptoms among migraineurs people

(Hagen et al. 2002). Again, a US study revealed how men are more likely to have somatic comorbidity than women, who have more psychiatric comorbidity (Tietjen et al. 2007). However, two studies have reported, on the contrary, how men are more prone to show anxiety or depressive symptoms compared with other non-migraineurs men (Lampl et al. 2016; Victor et al. 2010). All these differences could be due to methodological differences among the epidemiologically studies. Despite these contrasting pieces of information, an increasing amount of evidence has identified migraine in women, as a risk factor for comorbidity with vascular pathologies (M E Bigal et al. 2010; Kurth et al. 2016, 2009).

TTH headache epidemiology – this is the most prevalent primary headache. It has been estimated that nearly three billion individuals suffered from migraine or tension-like headaches in 2016. The Global Burden of Disease (GBD) indicated that tension-type headache is the third most prevalent cause of disease, and migraine is the sixth. For TTH, the global age-standardized prevalence was 26.1% overall: 30.8% for women and 21.4% for men. In 2016, the age-standardized prevalence of tension-type headaches was highest in Brazil and Afghanistan. The lowest prevalence was in China disorder (GBD 2016 Headache Collaborators 2018).

There are several studies estimating TTH prevalence. The largest European studies (Russell et al. 2006) evaluated over 33,000 Danish twins aged 12 to 41 years old. The 1-year prevalence was 83.5% (78.9% in men and 92.5% in women) for TTH. 63.5% of overall patients had infrequent ETTH, 21.6% had frequent ETTH, and 0.9% had CTTH. In this study, women are more likely to have frequent ETTH and CTTH. In the case of ETTH the prevalence increases until age 39 and then decline both in man and women. On the other hand, CCTH is rare in early adolescence and the prevalence increases until age 39 and then declined (Russell et al. 2006). According to data reported by the GBD (GBD 2016 Headache Collaborators 2018), in the TTH, a peak in prevalence and YLD rate occurred between the ages of 35 and 39 years (Figure 2). In both sexes, the percentages of all YLDs were highest in the group aged 15–49 years (1.3%), but were also high in children aged 5–14 years (0.6%), in individuals aged 50–69 years (0.7%), and in the elderly (ie, ≥70 years; 0.3%). In women between ages 15 and 49 years, tension-type headaches caused 2.9 million YLDs in 2016.

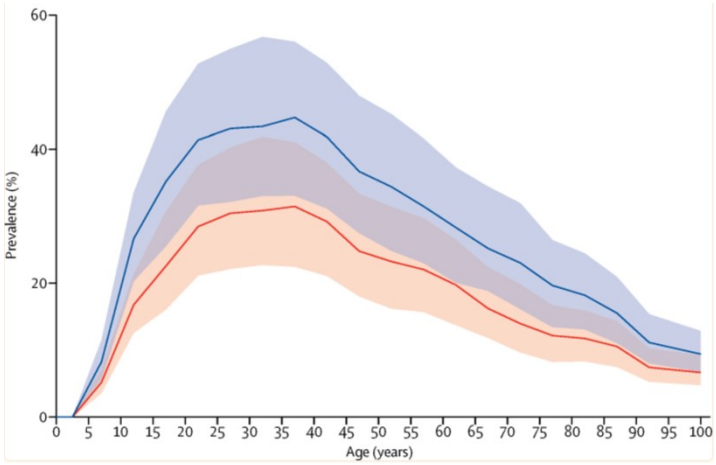

Figure 2. Tension-type headache by age and sex, 2016. Reproduced from (GBD 2016 Headache Collaborators 2018). Prevalence is expressed as the percentage of the population that is affected by the disease. Shaded areas show 95% uncertainty intervals. Values are plotted at the midpoint of 5-year age categories.

Both in men and in women, the TTH headache could be accompanied by comorbidities, such as psychiatric comorbidity including anxiety, depression, and sleep disturbances (i.e. insomnia) (S Ashina et al. 2017; Ødegård et al. 2013, 2010; Kim et al. 2017; Song et al. 2016). Anxiety and depression are often associated with the frequency and severity of TTH attacks (Mitsikostas and Thomas 1999). Other comorbidities associated with TTH are somatic, such as neck pain and low back pain (Yoon et al. 2013; S Ashina et al. 2018; Sait Ashina et al. 2015). It was demonstrated also that TTH is associated with pain disorders, including migraine (Rasmussen et al. 1991).

Cluster headache epidemiology – is the most common and best-known TAC headache. Is rare compared to migraine, and because of that it is demanding an accurate estimate of the incidence of cluster headaches within the community but has a population prevalence of around 0.1%. This disorder, different from migraine and TTH, affects more men than women (Robbins and Lipton 2010). More detailed analysis of gender relationships by age of onset showed that the male-female ratio was highest among patients whose onset age was between 20 and 49 years; in episodic cluster headache (ECH) was 7.2:1, and in chronic cluster headache (CCH) was 11:1. This ratio was lowest at age 50, when the ratio was 2.3:1 in CH and 0.6:1 in CH. (Ekbom et al. 2002).

Unlike in migraine, no clear relationship has been established between cluster headaches and estrogens, in particular with oral contraception, hormone replacement therapy, menstruation, pregnancy, and menopause (Bahra, May, and Goadsby 2002; Manzoni et al. 1988).

MOH epidemiology – In GBD 2013 and GBD 2015, medication overuse headache was treated as a separate disorder, but in GBD 2016 it was considered a sequela of either migraine or tension-type headache (GBD 2016 Headache Collaborators 2018). MOH prevalence is about 2% among women and about 1% among men worldwide in industrialized countries (Lu et al. 2001; Colás et al. 2004; S.-J. Wang et al. 2006; Aaseth et al. 2008).

1.3 Pathophysiology of migraine

Research on migraine has come a long way in the last decades (Peter J Goadsby 2010): migraine has been better categorized and defined (ICHD-3 2018); imaging studies have shown that cortical-spreading depression is very likely the phenomenon underlying the migraine aura (J Olesen, Larsen, and Lauritzen 1981; J Olesen et al. 1990; Hadjikhani et al. 2001); the neurobiology of the trigeminal vascular system has been defined (M A Moskowitz 1984, 1991; Michael A Moskowitz 2007; Levy, Jakubowski, and Burstein 2004; Strassman, Raymond, and Burstein 1996; Peter J Goadsby 2005); several messengers involved in the mechanisms of onset and maintenance of migraine have been identified (Lassen, Thomsen, and Olesen 1995; J Olesen, Iversen, and Thomsen 1993; J Olesen, Thomsen, and Iversen 1994; Iyengar et al. 2019; De Logu et al. 2017, 2022); the importance of central sensitization (increased excitability of neurons in the central nervous system) of pain routes have been documented (R Burstein et al. 2000; Rami Burstein, Collins, and Jakubowski 2004). Despite all these findings, migraine pathophysiology continues to be poorly known (Headache Classification Subcommittee of the International Headache Society 2004). This chapter will try to resume the major theories and discoveries at the base of migraine, trying to give a clear picture of the current knowledge about the topic.

Theories behind the onset of migraine attacks in the years – several theories on the migraine onset have been proposed in the years. The first one was the *vascular theory* proposed by Wolff several years ago (Wolff HG. 1938; Ray and Wolff 1940).

According to this theory, the dilatation of cerebral vessels was long considered to be the cause of migraine pain. To assess this hypothesis several studies have been conducted during the years, both in experimental animals and humans. In humans the injection of migraine-evoking drugs has been associated with the use of magnetic resonance angiography (MRA) to monitor extracranial arteries dilatation. In studies with glyceryl trinitrate (GTN), GTN caused dilatation of extracerebral arteries both in healthy subjects and in migraine patients. Vasodilatation was greater in patients with migraine than in healthy controls (Thomsen et al. 1993). In healthy volunteers, GTN caused dilatation of extracerebral arteries and migraine occurs simultaneously (Tegeler et al. 1996) (Tegeler et al. 1996; Iversen et al. 2008) (J. M. Hansen et al. 2007). Similar results have been obtained using calcitonine gene related peptide (CGRP) as migraine trigger (Mohammad S Asghar et al. 2011) (M S Asghar et al. 2010) (Petersen et al. 2005). However, some studies disproved the migraine vascular theory: i.e. sildenafil-induced migraine was not associated with arterial dilatation (Kruuse et

al. 2003), while the vasoactive intestinal peptide leaded to marked extracranial vasodilation but did not cause migraine (Rahmann et al. 2008). Additionally, Ashina et al. (2011) reported no temporal correlation between maximal headache score and maximal vasodilatation. More importantly, an MRA study in patients with migraine without the use of migraine triggers, revealed no dilatation of extracranial arteries during spontaneous attacks (Amin et al. 2013). In conclusion, vasodilatation seems to be involved in migraine induction, but migraine can occur without vasodilatation, and vasodilatation can also occur without causing migraine.

In parallel with the vascular theory of migraine, the *neurogenic theory* of migraine has been developed. According to this, migraine is a neurological disease, and the changes in blood vessels that occur during a migraine attack are due to neuronal changes. This theory was proposed by the Brazilian neurophysiologist Leão in 1944 (Leao 1944). In this first publication, he discovered the Cortical Spreading Depression (CSD), that is a wave of excitation of the neurons in the cortex, followed by a period of hyperpolarization (Figure 3), and in the subsequent, he described a wave of dilatation of pial vessels all over the cerebral hemispheres concomitant with the CDS (Aristides A. P. Leo 1944). Later, in a third paper, he proposed that CDS might be related to migraine with aura (A A P Leo and Morison 1945).

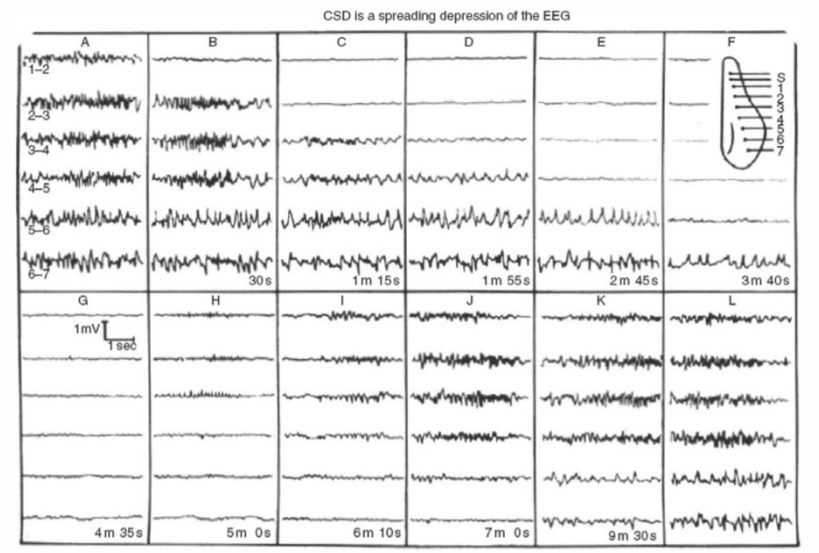

Figure 3. Leão's original illustration of spreading depression in the rabbit brain. EEG graph shows the cortical spreading depression (CSD) from Leão's 1944 paper. Reduction of electrical activity appears first at the region that has been stimulated and spreads out from there in all directions, involving successively more and more distant parts of the cerebral cortex.

In the following decades, CSD and its relationship with migraine were extensively studied in various animal models, leading to a more detailed understanding of its mechanisms (A. J. Hansen and Zeuthen 1981; Mutch and Hansen 1984; Dietz, Weiss, and Shuttleworth

2008). More recent studies suggest that CSD is responsible for the migraine visual aura (Tfelt-Hansen 2010; ICHD-3 2018; Bolay, Vuralli, and Goadsby 2019; Bolay et al. 2002), and particularly, Bolay et al. demonstrated for the first time how CSD participates in the activation of the trigeminovascular afferents, evoking a series of cortical meningeal and brainstem events consistent with the development of headache (Bolay et al. 2002). Even if CSD is considered to have this important role in the onset of aura, its role in migraine without aura is not understood.

Despite all these pieces of evidence, neither the vascular theory nor the neuronal theory are sufficient alone to explain the mechanisms and the complex biological events underlying the migraine attack. The complex interactions between the vascular system and sensory neurons, at the intracranial level, are important in the onset of migraine. This relationship was studied for the first time by Moskowitz in the '80s (M A Moskowitz 1984; Mayberg et al. 1981), which introduced the *trigeminovascular hypothesis* of migraine, drawing attention to the key role played by the trigeminal nerve and its vasoactive axonal projections, containing neuropeptides, on the meninges and its blood vessels. Today this is the most accredited theory.

The trigeminal nerve pathways – The trigeminal nerve is the 5th and largest cranial nerve and represents one of the most important anatomical and functional areas for the study of the pathophysiology of migraine. The trigeminal nerve emerges from the brain stem with two distinct roots and directs and penetrates into the Meckel cable or trigeminal cord, a structure located outside the blood-brain barrier (BBB). Within this is located the trigeminal ganglion or semilunar ganglion of Gasser, from which the three sensory branches of the trigeminal nerve depart: the mandibular division (V3), the maxillary division (V2), and the ophthalmic division (V1) (figure 7). These three branches innervate face, forehead, eyelids, nose, earcup, tongue, teeth, cerebral blood vessels, dura mater, and in the posterior area of the head and neck. In particular, V1 is responsible for the sensitive innervation of the superior skin of the face and cornea, the majority of the dura (anterior cranial fossa, falx, tentorium, venous sinuses) and brain proximal arteries. V2 is responsible for the innervation of the middle part of the face (bottom eyelid skin, nose, upper lip, sinuses, nasal and oral mucosa, upper gums, and teeth) and a small area in the dura mater. As opposed to V1 and V2, V3 innervates the lower-facial part (including gums and teeth, skin in the temporal region, part of the auricle, and lower lip) and a small part of the temporal dura mater. The

efferent motor fibers for masticatory muscles are also present in the mandibular division (**Figure 4**).

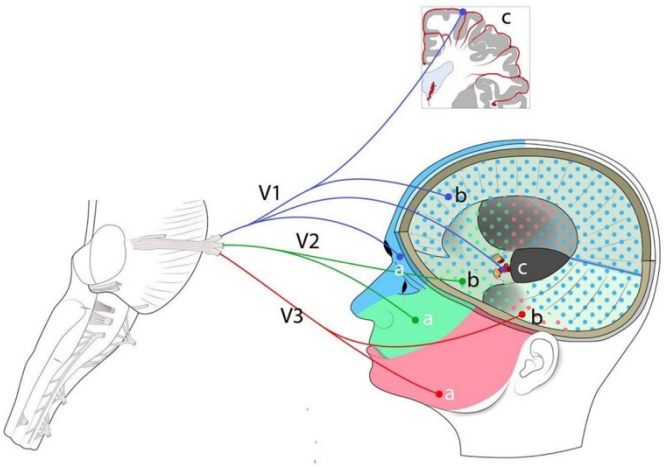

Figure 4. Trigeminal division. Picture taken from (Terrier, Hadjikhani, and Destrieux 2022). The three trigeminal divisions (ophthalmic or V1, maxillary or V2, and mandibular or V3) of the trigeminal nerve get afferents from the mucosa and skin (a) and the dura mater (b), the dural territory of V1 being the largest one. In addition, afferent fibers from the large cerebral and pial arteries (c), and probably from the "pia arachnoid" (trigeminovascular system), reach the V1

Each of the trigeminal branches incorporates many types of fibers, including those dedicated to touching, proprioception, pressure, vibration, and nociception from the face, and from the meninges and brain blood vessels (Joo et al. 2014; Miller et al. 2005) without a clear configuration of the fiber by modality. However, the fibers of the trigeminal neuro-vasculature that innervate the cerebral vessels and meninges are exclusively nociceptive and run within the V1 branch (Fontaine et al. 2018).

V1, V2, and V3 converge then in the trigeminal ganglion (Mai and Paxinos 2012; Terrier, Hadjikhani, and Destrieux 2022). These trigeminal neurons have two axonal projections, which extend in opposite directions: one toward the trigeminal divisions and the other up to brainstem nuclei (Figure 5) (Joo et al. 2014).

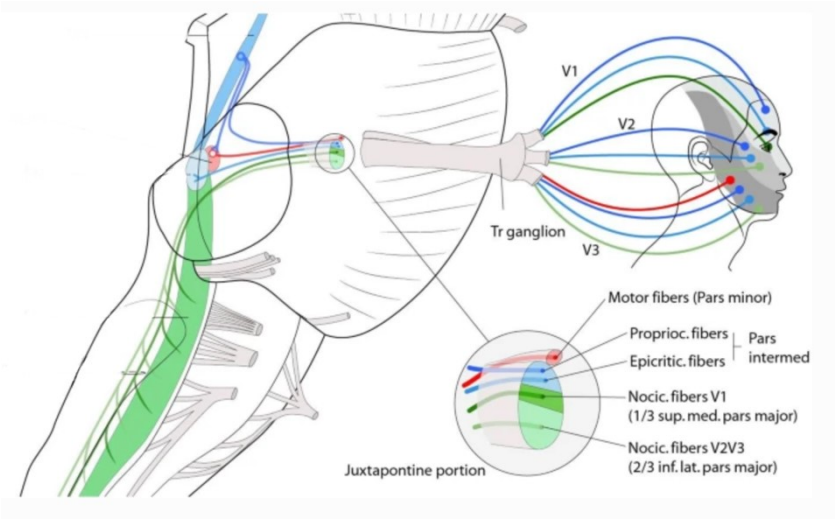

Figure 5. Picture taken from (Terrier, Hadjikhani, and Destrieux 2022). The three trigeminal divisions V1, V2, and V3 of the trigeminal nerve contain proprioceptive (dark blue), epicritic (light blue), and nociceptive (green) fibers. In V3 there are also motor (red) fibers for masticatory muscles.

Because of these anatomical characteristics, trigeminal neurons are thought to be able to conduct action potentials in both orthodromically (through the brainstem), and antidromically (towards branches of the trigeminal divisions) directions. This latter type of conduction usually occurs in migraine and mediates the peripheral release of inflammatory neuropeptides by the trigeminal system (Jes Olesen et al. 2009; Ramachandran 2018).

Once in the trigeminal ganglion, the fibers converge in the trigeminal root to the ventrolateral pons where they bifurcate to reach the different nuclei, that form the trigeminal nuclear complex which is a long column of cells located in the dorsolateral brainstem (Figure 6).

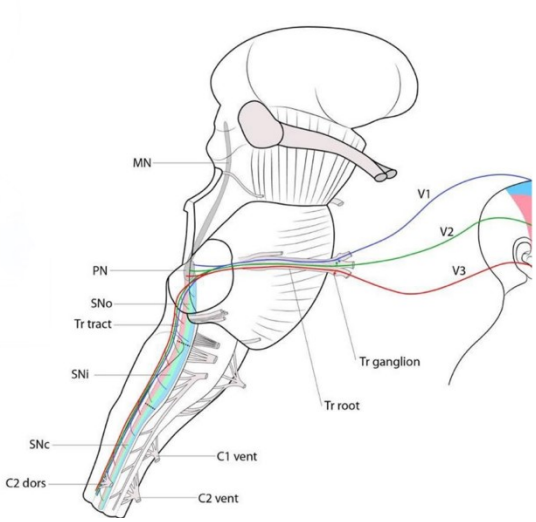

Figure 6. Picture taken from (Terrier, Hadjikhani, and Destrieux 2022). Trigeminal tract (Tr tract) and spinal trigeminal nucleus afferences and anatomy. Afferent fibers convey information to the three trigeminal divisions (V1, V2, and V3), that converge in the Trigeminal ganglion (Tr ganglion) and then in the trigeminal root (Tr root). This latter structure converges in the pons and their fibers bifurcate in the different nuclei: fibers for touch reach the principal nucleus (PN), proprioceptive fibers reach the mesencephalic nucleus (MN), nociceptive fibers enter the spinal trigeminal nucleus (SN). This latter nucleus is further divided in: pars oralis (SNo) plays a role in intraoral and dental sensation, such as intraoral pain, but also epicritic signals from the face. These fibers are specialized in processing short-duration information after nociceptive stimuli such as formalin; ii) pars interpolaris (SNi), which receives cutaneous nociceptive fibers or low-threshold mechanonociceptor. Its function remains unclear and iii) pars caudalis (SNc) responsible for pain and thermal perception of the facial and cranial tissues. In this part, it terminates most of the small diameter myelinated (Aδ) and unmyelinated (C) fibers. This part is most involved in processing tonic signals than short-duration nociceptive stimuli (i.e. by noxious chemicals).

From the PN and SN, secondary neurons go to innervate the thalamus at the level of the ventro-posteromedial (VPM) and ventro-posterolateral (VPL) nuclei, through both the ventral (VTT) and dorsal (DTT) trigemino-thalamic tracts. On the other hand, neurons from caudal SN, involved in extreme thermal perception, reach the thalamus crossing midline to join the spinothalamic tract (Figure 7).

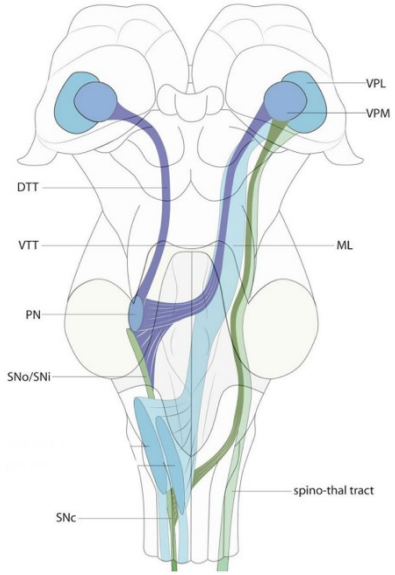

Figure 7. Picture adapted from (Terrier, Hadjikhani, and Destrieux 2022). PN and SN nuclei reach the thalamus at the level of ventro-posteromedial nuclei (VPM) and ventro-posterolateral nuclei (VPL) in three ways: the ventral trigemino-thalamic tract (VTT), which crosses the midline to join the median lemniscus (ML) before reaching the thalamus. The dorsal trigemino-thalamic tract (DTT) reaches the ipsilateral thalamus. Finally, neurons from SNc cross the midline to meet the spinothalamic tract and then reach the thalamus.

Some studies show how the thalamocortical circuits are involved in the onset of the premonitory phase of migraine (de Tommaso et al. 2014). Structural and functional studies prove changes in thalamic and thalamocortical activity in migraineurs than in healthy patients both during and between the attacks (Hodkinson et al. 2016; Coppola et al. 2016). Others define the thalamus as an important mediator of cutaneous allodynia (T. Wang et al. 2015) and responsible for the aggravation of photophobia (Noseda et al. 2016). Taken together, all these findings prove that changes in thalamic and thalamocortical activity are responsible for the occurrence of aberrant sensory processing during a migraine attack. Even if the sensation of pain becomes conscious in the thalamus, for more accurate localization and discrimination of intensity and quality of the noxious stimuli, is necessary further processing that takes place at the level of primary and secondary somatosensory (S1/S2) cortices (Augustine 2017).

Activation of trigeminovascular pathway – It is believed that the headache phase of a migraine crisis begins with activation of nociceptors innervating pial, dural blood vessels, and arachnoid, as well as major brain arteries and sinuses (Jes Olesen et al. 2009). These nociceptors consist of nonmyelinated (C-fibers) and myelinated (Aδ-fibers) axons containing vasoactive neuropeptides such as substance P (SP), calcitonin gene-related peptide (CGRP), pituitary adenylate cyclase-activating polypeptide (PACAP), vasoactive intestinal peptide (VIP) and other non-peptide neurotransmitters such as glutamate (Messlinger et al. 1993; Keller and Marfurt 1991; Levy and Strassman 2002; Sajedeh Eftekhari et al. 2013). The meningeal vessels are also innervated by parasympathetic fibers, which contain acetylcholine, nitric oxide (NO), and VIP (Roloff et al. 2016). The local stimulation of meningeal afferents with electrical stimuli or with chemicals (i.e. 5-HT, prostanoids, H^+, bradykinin, capsaicin, mustard oil) leads to the calcium-dependent relies of neuropeptides such as CGRP, SP, PACAP, and VIP. These lead to a cascade of local events including vasodilatation, plasma extravasation, mast cell degranulation, and chemotaxis of white blood cells. This phenomenon is named neurogenic inflammation and its persistency is proposed to be involved in the peripheral sensitization process and in the central sensitization process typical of migraine pathology. Sensitization leads to increased spontaneous neuronal activity and a heightened response to innocuous cephalic and extracephalic stimuli (Rami Burstein, Noseda, and Borsook 2015). Sensitization clinically manifests as cutaneous allodynia, hyperalgesia, and secondary hyperalgesia (Woolf 2018, 2011). For recent reviews in the field see (Iyengar et al. 2019; Terrier et al. 2021). Among neurotransmitters, CGRP at the trigeminal level is considered a principal mediator of migraine. Approximately one-half of human trigeminal ganglia neurons express CGRP, while CGRP receptor components are expressed in about one-third of trigeminal Aδ fibers, in vascular smooth muscle cells and in trigeminal ganglion satellite cells (S Eftekhari et al. 2010). In this regard, a recent study investigated the role of CGRP in peripheral sensitization by addressing the CGRP receptor components (CLR/RAMP1) expressed on trigeminal glial cells (Schwann cells) (De Logu et al. 2022). This study proposes the CLR/RAMP1 expressed in trigeminal Schwann cells as the key mediator of enhanced sensitivity in primary sensory neurons. According to this study, CGRP released from trigeminal cutaneous afferents caused the activation of its receptor in Schwann cells. CLR/RAMP1 is internalized in endosomes whit a consequent increase of cAMP which stimulates PKA. This mechanism results in nitric oxide synthase activation. The consequent release of NO targets the transient receptor potential cation channel, subfamily A, member 1 (TRPA1) in Schwann cells, which elicits radical oxygen species (ROS) generation. ROS triggers TRPA1 on adjacent C- or Aδ-

fibers afferents causing periorbital allodynia (Figure 8). Of note, the events able to activate meningeal neurogenic inflammation are still not completely understood. Interestingly, some authors support the hypothesis CSD may cause neurogenic meningeal inflammation and later activate the trigeminal system (Zhang et al. 2011, 2010; Spekker et al. 2021; Bolay et al. 2002). In particular, CSD is associated with increased levels of ATP, glutamate, and potassium that diffuse toward the cortex and can activate meningeal nociceptors (Bolay et al. 2002; Terrier et al. 2021). Alternatively, during CSD, cortical neurons can induce the synthesis of prostaglandins and cytokines in glial cells that activate trigeminal receptors at the level of pia and pial arteries, causing an orthodromic activation of the trigeminal nerve, which transmits the nociceptive signal to the CNS (Terrier et al. 2021). Despite these hypothesis the nature and mechanisms of biological events leading to episodic activation of the trigeminovascular pathway in migraine remain incompletely understood and still controversial.

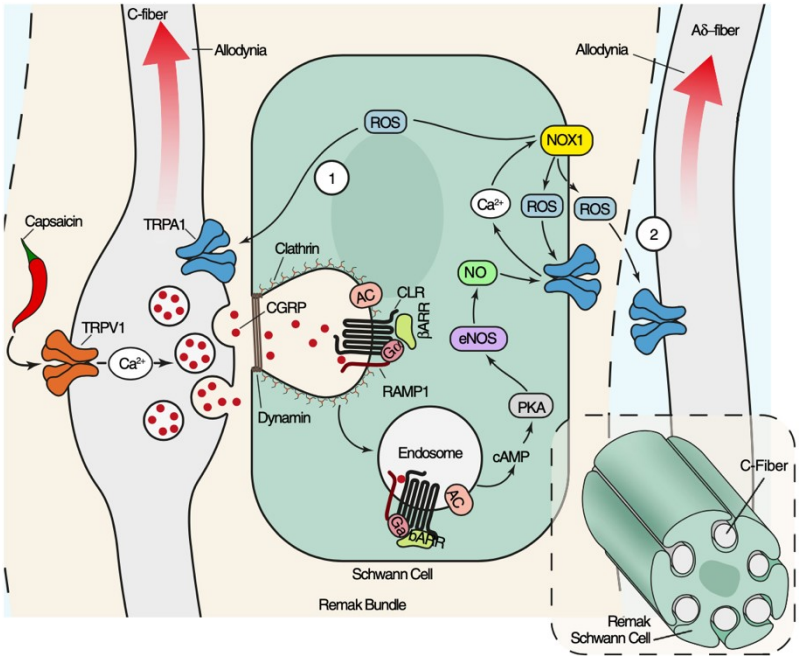

Figure 8. Figure taken from (De Logu et al. 2022). Schematic representation of the signaling pathway responsible for the peripheral sensitization elicited by CGRP release.

1.4 Pharmacology of migraine: current treatments

Current drug therapies for the treatment of migraines can be divided into two macro-categories: symptomatic (or acute) therapy and preventive therapy.

Symptomatic or acute treatment – The main purpose of acute therapy is to exercise rapid control over symptoms, and decrease functional disability reducing the duration of attacks and their severity. The US Headache Consortium has described the goals for the acute migraine treatments, consistent in i) treating crises promptly and systematically without recurrence; ii) restoring the patient's well-being and reducing subsequent use of other drugs; iii) minimizing the use of symptomatic medications; iv) being cost-effective for overall management; vi) have minimal or no adverse events. For the acute treatment of migraine specific and non-specific treatments are available (Ong and De Felice 2018).

Specific pharmacological treatments

- *Drugs targeting the 5-HT system (ergot alkaloids, triptans, and ditans)*

  Ergot alkaloids such as *ergotamine* (approved in 1976) have been used since the 1970s for the symptomatic treatment of acute migraine. Dihydroergotamine (DHE) is prescribed for the treatment of migraine with or without aura, and for cluster headache episodes. DHE interacts with a high-affinity with 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptors. Its efficacy is believed to be due to i) the activation of 5-HT_{1D} at the level of intracranial blood vessels with subsequent vasoconstriction, or ii) the activation of 5-HT_{1D} receptors on sensory nerve endings of the trigeminal system and consequent inhibition of the release of pro-inflammatory peptides (Silberstein and Kori 2013). Its use is limited to those patients unresponsive to other therapies such as triptans. A nasal power formulation of DHE (STS101) is currently in Phase 3 clinical trial as acute anti-migraine agent (ASCEND, ClinicalTrials.gov Identifier NCT04406649) (Ong and De Felice 2018).

  Triptans have been chosen as first-line therapy for almost 25 years. They are analogs of serotonin and stimulate the 5-$HT_{1B/1D}$ receptors at the level of cranial blood vessels and nerve endings, relieving pain through the inhibition of the release of CGRP and substance P. Sumatriptan was the first compound of this class, synthesized with the aim of identifying a molecule with properties similar to ergot but with fewer side effects at the vascular level. Triptans are generally well tolerated in patients who do not have concomitant vascular pathologies, but they excessive use could be the cause of the onset

of migraine known as medication-overuse headache (MOH). They are contraindicated in patients with cardio-vascular disorders, hypertension, intestinal ischemia and during pregnancy (Ong and De Felice 2018). Other triptans drugs used as anti-migraine agents are Zolmitriptan, approved by FDA in 1997, and Rizatriptan, approved by the FDA in 1998. Triptans have proven to be effective in the treatment of menstrual migraine attacks. Efforts have been done during the years to develop new acute migraine treatments that work by targeting the trigeminal pathways avoiding the vasoactive 5-HT receptors (Oswald and Schuster 2018).

This lead to the development of the 5-HT_{1F} agonists *Ditans.* Lasmiditan is a highly selective 5-HT_{1F} agonist and was approved in 2019. Ditans are structurally different from triptans: triptans have an indole structure that ditans replace with a pyridine-piperidine scaffold. While triptans bind 5-HT_{1B} and 5-HT_{1D} receptors without selectivity, causing direct vascular vasoconstriction, ditans are selective for the 5-HT_{1F} receptor, performing an antimigraine action acting mainly at the neuronal level, not causing vasoconstrictor effects (Zobdeh et al. 2021). Evidence from the preclinical study demonstrates that Lasmiditian inhibits the release of CGRP from the presynaptic membrane of the trigeminal system blocking plasma protein extravasation (Labastida-Ramírez et al. 2020). Lasmiditian is well tolerated and represents a promising therapy for patients with cardiovascular risk factors. Lasmiditan is currently in a Phase 3 trial testing safety and efficacy as acute migraine treatment in childrens (ClinicalTrials.gov Identifier NCT04396574).

- *Drugs targeting the CGRP system (Gepants)*

  They represent a novelty in the acute treatment of migraines and a possible alternative to triptans for those patients for whom they are contraindicated or ineffective. Gepants have been investigated regarding their effect on both acute migraine treatment and migraine prevention. They are selective and competitive small molecules antagonists of the CGPR receptor. Gepants were recently approved both for acute and for preventive migraine treatment. Two gepants have been approved by FDA for acute migraine menagment: Ubrogepant in 2019, and Rimegepant in 2020. This latter compound has been approved also by EMA in 2022. Ubrogepant (Ubrelvy™), is orally administered and is a potent, highly selective, competitive CGRP receptor antagonist developed by Allergan, under a license from Merck & Co. Ubrogepant is the first-line drug for the treatment of migraine in the class of oral CGRP antagonists (Scott 2020). The most

common adverse events reported are nausea, somnolence, and dry mouth with a frequency rate below 5% (Ailani et al. 2020). Rimegepant is the second drug of this class, approved by FDA in February 2020. Gepants can be used as a first-line anti-migraine treatment in patients with increased risk for cardiovascular events, or in those patients with documented cardiovascular disease, and as a second-line treatment where treatment with triptans has failed (Do, Guo, and Ashina 2019).

Non-specific pharmacological treatments

- *Non-steroidal anti-inflammatory drugs* (*NSAIDs*) – are medications commonly used to treat pain, inflammation, and fever. Generally, they have anti-inflammatory action at a lower dosage than necessary to show antipyretic and analgesic activity. NSAIDs are all organic acids and this promotes their accumulation in sites where a drop in pH is detected, such as sites where an inflammatory process is ongoing. They are divided into different chemical classes and, by virtue of their chemical heterogeneity, have different pharmacokinetic characteristics (Ong and De Felice 2018). They work by inhibiting the enzyme COX1 (cycloxygenase 1) and/or the enzyme COX2 (cyclooxygenase 2) and consequently the synthesis of prostanoids, which stimulate inflammatory processes and pain transmission. COX1 is a constitutive enzyme unlike COX2, an inducible enzyme that, in case of an inflammatory process, leads to an overproduction of prostanoids, for that reason inhibition of COX2 lead to an analgesic, anti-inflammatory, and antipyretic effect, on the other hand, blocking COX1 leads to ulcerations and gastrointestinal bleeding (Silberstein and Stirpe 2014). In the first-generation NSAIDs, the selectivity for COX1 or COX2 is variable and incomplete, and side effects such as gastric ulcers may occur. Second-generation NSAIDs, although selective for COX-2 and with fewer gastrointestinal side effects, have more adverse cardiovascular side effects. For that reason, this class of drugs is contraindicated in patients that suffer of peptic ulcer, inflammatory bowel disease, gastic bypass, allergies to salicylic acetyl acid, kidney dysfunction, heart failure or during pregnancy. Like triptans, the abuse of NSAIDs lead to MOH (Ong and De Felice 2018).

- *Acetaminophen* – acetanilide and phenacetin are the two active metabolites of paracetamol with antipyretic action. Its mechanism of action has not yet been fully clarified but probably it inhibits the synthesis of prostaglandins (Graham and Scott 2003). Compared to NSAIDs, it has less gastrointestinal activity because it does not

directly interfere with COX functions outside the central nervous system. Its use is well-documented for the treatment of migraine (R B Lipton et al. 2000; H. C. Diener et al. 2005). It can also be used in combination with other molecules: in particular, the association of paracetamol/ aspirin/ caffeine has good effectiveness (H. C. Diener et al. 2005). Opioid combinations, such as paracetamol/codeine must not be used routinely in the treatment of migraine due to the risk of developing a MOH. When opioid use is needed, combination with tramadol is a preferred option (Silberstein et al. 2005). Acetaminophen is the painkiller of choice for the treatment of mild to moderate migraine seizures during pregnancy. Acetaminophen combined with codeine is also considered to be relatively safe during pregnancy. (Worthington et al. 2013).

- *Dopamine Antagonists (The Antiemetics)* – Drugs belonging to this category are useful for the treatment of both migraine and nausea that occurs during a migraine attack. Among these, we can find Metoclopramide (Ong and De Felice 2018), Chlorpromazine and Prochlorperazine. All these medications can be administered in combination with other drugs commonly used to treat migraine attacks. A number of adverse reactions can be associated with dopamine blockages, such as drowsiness and weight gain, postural hypotension, sedation, blurred vision, cardiac arrhythmias, and urinary retention. Moreover, dopamine blockage may also lead to extrapyramidal symptoms such as drug-induced pseudo-parkinsonism, delayed dyskinesia, akathisia, dystonia, and tremors. An increase in prolactin release with a consecutive sexual malfunction, galactorrhea, and infertility may also occur. However, such events are uncommon with the intermittent oral dosage used for treating migraine attacks (Ong and De Felice 2018).

- *Opioids* – their use as anti-migraine drugs is controversial. Opioids modulate pain transmission through the trigeminovascular complex and have neither vasoconstrictive nor anti-inflammatory effects (Ong and De Felice 2018). However, opioids have a high potential for abuse and can induce central sensitization and increase the risk of developing MOH. Because of this, their use should be considered when other therapies failed and after a careful assessment of the risk of abuse (Ong and De Felice 2018).

Preventive or prophylactic treatment – A preventative medication for migraine is considered effective if it reduces the frequency of migraine seizures by at least 50% in 3 months.

Specific drugs, that include monoclonal antibodies for CGRP (mABS) and Gepants are available, as well as non-specific treatments, such as beta-blockers, calcium antagonists, antiepileptic drugs and botulinum toxin (Zobdeh et al. 2021).

- *Gepants and anti-CGRP monoclonal antibodies (mAbs)* – considering the critical role of CGRP in migraine onset, CGRP mAbs represent the first preventive medicines specifically developed for the treatment of migraine. These are mAbs directed toward both CGRP or its receptor (Manoukian et al. 2019). Currently 4 CGRP mAbs have been approved for migraine prevention: Galcanezumab, Eptinezumab, and Fremanezumab are mAbs that target the neuropeptide CGRP (Melo-Carrillo et al. 2017), whereas Erenumab is a mAb that targets the CGRP receptor (Manoukian et al. 2019). Compared to Gepants, which are small-molecule CGRP antagonists, mAbs have a much higher target specificity, longer circulating half-life, and lower drug-drug interactions. With poor oral bioavailability due to gastric degradation, mAbs are administered parenterally (intravenous or subcutaneous) (F. Cohen et al. 2022). The American Headache Society recommend initiation of CGRP mAb upon failure of at least 2 standard oral migraine preventives (F. Cohen et al. 2022), to the other hand, recently updated European Headache Federation guidelines recommend, in patients who need preventive treatment, the use of mAbs as first line treatment option (Sacco et al. 2022). Anyway, a recent systematic review suggest that CGRP mAbs have a more favorable benefit-risk ratio than established treatments for episodic and chronic migraine (Drellia et al. 2021). Atogepant is the only CGRP small molecule receptor antagonist developed and approved for migraine prevention (Peter J Goadsby et al. 2020). CGRP mAb and Gepants did not show cardiovascular adverse effects (Peter J Goadsby et al. 2017; Silberstein et al. 2017). Erenumab caused no side effects in patients with angina pectoris (Depre et al. 2018) but hypertension has been included as warning for this mAb (Dodick et al. 2021). Side effects related to the chronic use of CGRP mAbs and Gepants are nausea, fatigue, constipation, urinary tract infections and upper respiratory tract infection.

- *β-adrenoceptors antagonists* – Propranolol has been shown to significantly reduce the frequency, severity, and duration of migraine attacks since the 1970s (H. C. Diener et al. 2002). Its mechanism of action is not fully understood but it seems to inhibit the production of NO by blocking inducible NOS (Dakhale et al. 2019). Timolol is another, non-selective, β-adrenoceptor antagonist used for migraine prophylaxis since 1978 (Stellar et al. 1984; Durley, Cubberley, and Thomas 1981), and is thought to block the

β-adrenoreceptor with a consequent decrease in the synthesis and release of noradrenaline. Another proposed mechanism is the inhibition of glutamatergic transmission, and the reduction of the spread of signals through the brain (Shields and Goadsby 2005).

- *Candesartan* – is a blocker of the angiotensin II AT1 receptor, initially developed as an antihypertensive agent (Mizuno et al. 1992), and since 2003 used for migraine prophylaxis (Owada 2004). Its mechanism of action consists of the inhibition of the excessive vasoconstriction due to 5-HT release and inhibition of neurogenic inflammation.

- *Antiepileptic* – VPA (2-propylpentanoic acid) is the first-line therapy for migraine prophylaxis since 1996 (Cutrer et al., 1997), especially in the case of particularly frequent or chronic headaches. Its mechanism of action include the inhibition of GABA transaminase, and blockade of the sodium channels and T-type calcium channels (Zobdeh et al. 2021). Another drug in this class is topiramate, and its efficacy seems to be due to its ability to interact with different structures: i) it blocks channels at Na^+ dependent voltage, and thus decreases the frequency of activation of action potentials, ii) it inhibits the activity of glutamate on AMPA/kainate receptors; iii) inhibits trigeminal afferents by a GABA-mediated mechanism; iii) inhibits the release of CGPR from trigeminal neurons; iv) has an inhibitory effect on Ca^{2+} channels. Major side effects include: paresthesia, fatigue, drowsiness, nausea, weight loss, and cognitive disorders (Zobdeh et al. 2021).

- *OnabotulinumtoxinA* – OnabotulinumtoxinA is the accepted type for the treatment of migraine (M. Ashina, Buse, et al. 2021). It seems to modulate the release of several neurotransmitters: CGRP, substance P, serotonin, glutamate, GABA, noradrenaline, dopamine, and glycine, reducing pain in several conditions (Aurora et al. 2010; H. C. Diener et al. 2010). The mechanism by which it performs this action sees its effect at the level of peripheral nerve terminals, where it prevents the fusion of vesicles content neurotransmitters and neuropeptides, interfering with the formation of the protein SNARE complex (Rami Burstein et al. 2020). In clinical practice, botulinum toxin type A is administered according to a standardized injection protocol, every three months. However, about a third of patients do not respond to treatment (Zobdeh et al. 2021).

Despite the different drug therapies currently available, it should be noted that a large number of migraine patients are not satisfied with their therapy both for problems of effectiveness and tolerability (M. Bigal et al. 2007). A recent study (Orlando et al. 2020) highlighted that among patients who received prophylactic treatment, 73.8% discontinued the cure, while another (Hirata et al. 2020) demonstrated that 42.2% of migraine patients in cure whit acute treatments were unresponsive. This is why research to identify new therapeutic targets for the development of innovative, effective, and well-tolerated anti-migraine drugs is important and necessary.

1.5 Animal models of migraine

There are several validated mouse migraine models that are fundamental for studying disease pathophysiology and evaluating innovative pharmacological targets. A good animal model must have external validity, meaning that the results of the animal experiment can be generalized to other animal populations, including humans. The results obtained with a good animal model should therefore be translated into clinical applications (translational research). There are several differences between animal models and the clinical experience of patients, which limits the ability to translate the results of animal experiments. First, pain is often studied in young, healthy, and genetically similar male animals. This contrasts with the clinical situation in which pain occurs predominantly in women, middle-aged or elderly patients, with comorbidities, polypharmacy, and with heterogeneous genetic background. Secondly, animals do not effectively simulate the multidimensional nature of clinical pain conditions, which are influenced by complex psychological components, social parameters, educational level and environmental factors. Thirdly, animal models do not have the degenerative nature of chronic human diseases progressing over the years rather than weeks, as is the case in most laboratory experiments. Finally, pain cannot be measured directly in animal models; researchers must rely on surrogate behaviors, while patient pain is measured through self-reporting (Vierck, Hansson, and Yezierski 2008).

An animal model has external validity when three criteria are met. These are a construct, face, and predictive validity. Construct validity refers to the biological mechanisms underlying the animal model, which must be as similar as possible to the human biological mechanisms of the desease. An exemple of this is the use of mice with a genetic mutation to increase the vulnerability to the disease in humans (Nestler and Hyman 2010).

Face validity indicates that the animal model reflects the same signs as human patients. For example, in an animal migraine model, the animals should show some symptoms, such as allodynia or photophobia, that are present also in humans when they have a migraine attack. Finally, predictive validity refers to the fact that the animal model is responsive to known human therapies or to agents able to worsen the pathology in humans.

In vivo migraine studies are usually conducted using predominantly mice and rats. In all in vivo migraine models it is possible to identify i) a stimulus of various nature inducing migraine attack in the animal; ii) a useful measure to quantify the extent of the attack.

Direct stimulation of the trigeminal nerve – 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.

The first method involves the use of electric probes on anesthetized animals (Knyihar-Csillik et al. 1995). This technique has been particularly useful for the study of substances released during the migraine attack. For example, it has been shown that electrical stimulation of the trigeminal ganglion causes release of CGRP from perivascular meningeal affections, and this effect is inhibited by the treatment with classical anti-migraines such as triptans (Knyihar-Csillik et al. 1995). Electrical stimulation of the trigeminal meningeal nerve endings was instead indispensable both to identify the areas of the central nervous system important for the processing of migraine pain and to study the electrochemical responses to drugs (Buzzi et al. 1991). These methods are a robust model, and highly predictive of translational efficacy. Despite their high predicitivity, these methods are invasive and require the animal to be anesthetized for all the duration of the experiment.

Another strategy is the administration of inflammatory substances directly to the meninges. These substances can be applied individually or as a cocktail in a preparation called "inflammatory soup", in which are present histamine, serotonin, bradykinin, prostaglandin E2, capsaicin, acid or basic pH buffer, cytokines, and the Freund's adjuvant (Harriott et al. 2019). Administration of these substances to the dura mater causes activation and sensitization of both the trigeminal endings in the meninges and the central neurons in the trigeminal nucleus (Strassman, Raymond, and Burstein 1996). This method offers the advantage of the awake animal with the possibility to perform behavioral tests (i.e. allodynia tests with von Frey filaments) (Harriott et al. 2019).

Exogenous administration of algogenics substances in vivo – one of the principal characteristics of migraine is that various triggers are capable to initiate an attack, and this peculiarity is widely used during experiments in which several chemical triggers are used to evoke migraine (M. Ashina et al. 2017; M. Ashina, Hansen, and Olesen 2013). Among these, NO donors, including GTN given intraperitoneally (Marone et al. 2018), are the more frequently used. Importantly for the translational aspect, GTN is able to trigger migraine in patients (I Christiansen et al. 1999; Afridi, Kaube, and Goadsby 2004). Another substance widely used to induce migraine in mice and rats is the peptide CGRP, which is able also to

induce some common migraine signs such as photophobia, periorbital hypersensitivity, and spontaneous pain when given both intraperitoneally and subcutaneously (Rea et al. 2018; De Logu, Landini, et al. 2019; Mason et al. 2017). Other substances used are adrenomedullin, amylin, PACAP, VIP, histamine, and PGE_2, all able to induced periorbital mechanical allodynia in a recent study performed by De Logu and colleagues (De Logu, Landini, et al. 2019).

Experimental readouts: stimulus-evoked pain-like behavior – the most used behavioral evaluation in rodents is mechanical allodynia. Activation of the nociceptive system due to migraine attacks alter the threshold of pain to mechanical and thermal stimuli, producing amplified responses to harmful stimuli (hyperalgesia) and/or nociceptive responses to harmless sensory stimuli (allodynia). They are characteristic behaviors found both in animals where migraine has been caused, and in migraine patients during the attack and this gives the models high face validity. Manual Von Frey filaments are used for the evaluation of allodynia in rats and mice, and both periorbital and plantar allodynia can be evaluated with these methods (Harriott et al. 2019). Mechanical allodynia could also be evaluated through the use of electronic Von Frey. This system operates whit the same principles as the manual filaments, but in this case, an unbending filament is applied with increasing force until a nociceptive response from the animal. The advantages are multiple: experimental time is reduced, the force that caused a response is precisely determined, and fewer applications of the filament are necessary than the manual. Another way to evaluate mechanical hyperalgesia is the Randall-Selitto test, or paw pressure test, especially used in rats than in mice. Increasing pressure is applied through the use of specific apparatus on the dorsal or plantar surface of the hind paw and the end-point is considered a withdrawal response (Deuis, Dvorakova, and Vetter 2017).

It is possible to evaluate the hypersensitivity in the animal also towards thermal stimuli (both hot and cold). To assess hypersensitivity to heat the tail-flick test or the hot plate test can be used. In the first, animals are restrained and a heat stimulus is applicated to the tail. The time spent to “flick” the tail is recorded. The heat stimulus applied could also be radiant heat, or hot water, where the tail is immersed in a water bath with a temperature between 46° and 52°C, and the time spent to withdraw the tail is recorded (Deuis, Dvorakova, and Vetter 2017). The hot plate is another test used to evaluate the hind paw response to hot stimuli. In the conventional hot plate test mice are placed on a metal surface at a temperature between 50° and 55°C, and response latency is recorded. If no nociceptive behavior is observed the animal is removed from the hot surface in order to avoid tissues damages. On the other hand,

in the dynamic hot plate test the temperature increases gradually, starting from a non-noxious temperature of 40°C, until the first nociceptive response. Another test is the Hargreaves test in which is measured the heat threshold of the hind paw of mice through the application of a radiant or infrared heat stimulus at a temperature between 46°C – 52 °C. The time spent to to withdraw the paw is detected (Figure 9).

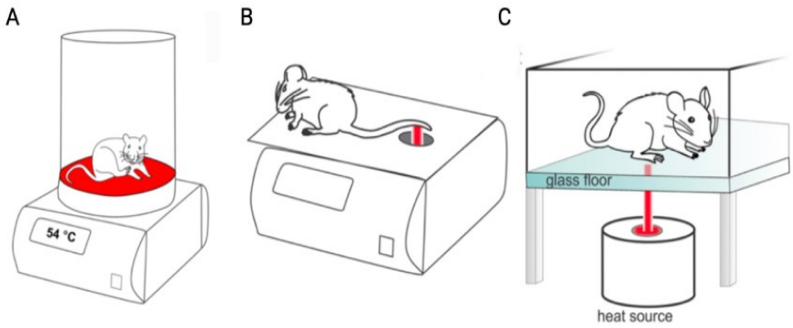

Figure 9. Picture taken from (Deuis, Dvorakova, and Vetter 2017). Methods used to assess pain evoked by heat in rodents. (A) Hot plate test; (B) Tail flick test; (C) Hargreaves test (Deuis et al. 2017)

The acetone evaporation test can be used to evaluate allodynia to cold stimuli. The test consists in the application of acetone on the plant of the animal's hind leg or in the periorbital region. The perceived temperature varies from 15 to 21 degrees and depends on the temperature of the room, skin and the amount of acetone applied. The duration, number or intensity of the nociceptive responses are then assessed. Another test that allows to evaluate the allodynia due to cold stimuli is the cold plate test, which consists in placing the animals on a plate set at a temperature ranging from -5 ºC to 15 ºC. The time needed to summon a nociceptive response is then evaluated (e.g. Licking, shaking or jumping) (Deuis, Dvorakova, and Vetter 2017). Similarly, in the cold plantar test, mice are placed in cages with glass floors and the nociceptive stimulus in this case is given by the application on the glass itself of an ice stick. Thermal allodynia is always evaluated based on the time it takes the mouse to retract the leg (Figure 10).

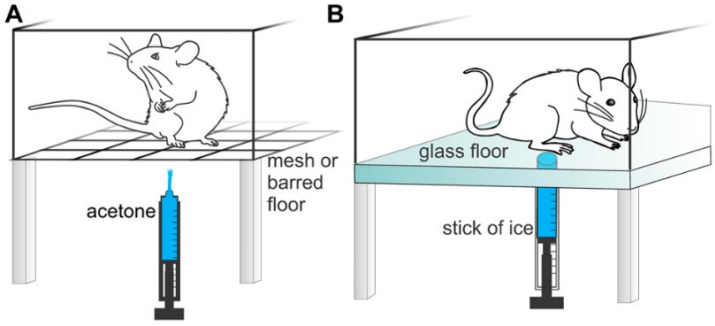

Figure 10. picture taken from (Deuis, Dvorakova, and Vetter 2017). Methods used to assess cold-induced allodynia. (A) Acetone evaporation test; (B) Cold plate test

Photophobia is another characteristic symptom of migraine patients. The tool mainly used to measure photophobia in rodents is the light dark box (Figure 11), that is a plastic box

where a compartment is illuminated (transparent walls) and one is completely dark (black walls). The animals are monitored and the time spent in each compartment is measured. A mouse that spends less time in the illuminated compartment than the controls has an aversion to light. Note that this test is also used to evaluate anxious-type behavior in rodents. Anti-anxiety medication increases the time the mouse spends in the illuminated compartment. This could be a confounding factor for anxiolytic compounds with putative antimigraine effect.

Figure 11. Picture taken from https://app.biorender.com. Light-dark apparatus.

Experimental readouts: non-stimulus evoked pain-like behavior – During the painful event, natural rodent behaviors such as exploration, locomotion, or food and water consumption may decrease. These behaviors are also found in patients suffering from migraines. The locomotor activity of the animal can be evaluated exposing the animal to a new environment (open field), or taking advantage of the natural propensity of the rodents to run on the wheel positioned in the home cage (Kandasamy, Lee, and Morgan 2017; Kandasamy et al. 2018) (Figure 12). It is worthy of note that some research groups reported increased rat locomotor activity after GTN administration (Sufka et al. 2016), suggesting that the reduction in rodent locomotor activity is not a robust migraine sign replicated across different laboratories.

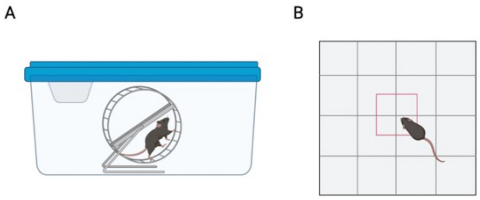

Figure 12. Picture was taken from https://app.biorender.com. Front image of the apparatus constituting the wheel for the stroke; B) Open field

A non-invasive method to assess spontaneous behavior associated with nocicection is the use of the Grimace scale (Langford et al. 2010). This is a scale that evaluates the intensity of pain in the rodent based on certain facial marks such as the closing of the eyes, the orientation of nose or ears, the swelling of the cheeks and nose, the position of the mustache (Figure 13). The Grimace scale has been used from some research group to assess the severity of migraine attack in rodents (S. Cohen and Beths 2020; Langford et al. 2010),

anyway its use is limited to few research articles and the possibility to use the Grimace scale with black mice is still debated.

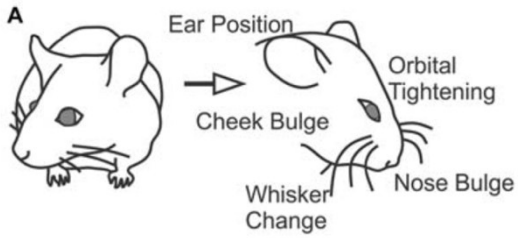

Figure 13. picture taken from (Deuis, Dvorakova, and Vetter 2017). Grimace scale: facial expression allows to evaluate the severity of pain based on different facial characteristics

1.6 New drug targets for migraine

Many patients are not satisfied with their treatment, and many do not tolerate or respond to actual acute therapy strategies. Some patients develop chronic migraine upon abuse of acute therapies such as triptans or opioids. People with frequent or chronic migraine are subjected to preventive therapies that include drugs from different classes such as antidepressants, anticonvulsants, calcium channel blockers, and others. Most of the time these therapies caused a lot of side effects. Recently, the discovery and use of mAbs targeting CGRP or its receptor have been shown to be effective as a preventive agent, but despite their efficacy and good tolerability, they have a very long half-life (3-6 weeks), and clinical consequences due to this long life, especially in patients with vascular risk factors, need to further safety study (Marcelo E Bigal, Walter, and Rapoport 2015). There is a real requirement for new safe and well-tolerated therapies that may fill this clinical gap and lead to the optimization of cures for patients.

1.6.1 Opioid receptor

Kappa-opioid receptor – The kappa opioid receptor is a Gi-coupled protein receptor and is widely expressed in structures involved in the control of mood states, anxiety, cognitive function, and modulation of rewards, such as the prefrontal cortex, amygdala, hippocampus, and hypothalamus., kappa agonists are used to treating peripherally disorders such as pruritus (Kumagai et al. 2010; Viscusi et al. 2021). Recently kappa agonists have received attention as better anti-nociceptive drugs than mu agonists (like morphine) because they do not induce respiratory depression (Viscusi et al. 2021; X. Wang et al. 2021). Despite this, the use of kappa agonists is limited because of their distinctive side effects that involve stress and aversion (Wee and Koob 2010; Dykstra et al. 1987), depression (Knoll and Carlezon 2010), sedation (Dykstra et al. 1987), diuresis (Meariman et al. 2022), and increases in serum prolactin, cortisol and adrenocorticotropic levels (Ko and Husbands 2022). Alternative strategies include the development of mixed opioid agonists (Atigari et al. 2021) or in order to avoid the central side effects, peripherally kappa agonists through structural modifications (Machelska and Celik 2018). Kappa agonists are currently under investigation as therapeutics for various kinds of pain such as acute pain (Escudero-Lara, Cabañero, and Maldonado 2021; Briggs 1998), inflammatory pain (Paton et al. 2017), neuropathic (Sounvoravong et al. 2004), and cancer pain (Edwards et al. 2018). Despite this, kappa agonists resulted inactive as antimigraine agents both in pre-clinical models (Williamson et al. 2001) and in humans (Williamson et al. 2001).

As said before, kappa is widely expressed throughout the central nervous system, including the amygdala and hypothalamus, two structures that seem to be highly involved in the onset of migraine. The activation of the hypothalamus seems to be involved in the premonitory phase of a migraine attack. One of the most common migraine triggers is stress (Maleki, Becerra, and Borsook 2012), and the relationship between stress and migraine is complex and still not understood. Repeated or chronic stress induces changes in the brain circuits (McEwen 2004) with consequent activation of the hypothalamus and release of corticotropin-releasing factor (CRF) that activate the CRF1 receptors in several brain regions (Griebel and Holsboer 2012; Koob 1999). Stress induces dynorphin release and activation of the kappa receptor in several brain regions, including the amygdala and the hypothalamus (Bruchas, Land, and Chavkin 2010; Land et al. 2008). This evidence led to the hypothesis that kappa antagonists can be effective preventive therapies for stress-related migraine. This hypothesis has been recently tested by Porreca's research group (Kopruszinski et al. 2021) in which they tested the standard kappa long-acting antagonist Nor-Binaltorphimine in blocking priming and preventing, or reversing umbellulone-induced allodynia in stressed animals. Moreover, in a recent work, Xie and colleagues (J. Y. Xie et al. 2017) demonstrate how kappa antagonism is able to prevent the expression of stress-induced cephalic and extracephalic allodynia in a model of sumatriptan-induced migraine in rats. They demonstrate also a consistent reduction of the plasma concentration of CGRP, produced by stress, in rats treated with two different kappa antagonists. In another work, Kopruszinski and colleagues (Kopruszinski et al. 2022) conducted a study in rodents that demonstrate the involvement of kappa receptor in the hypothalamus as a mediator of clinically-observed premonitory symptoms of migraine, such as increasing thirst and urination, but not pain responses. These findings give a better understanding of the premonitory phase, allowing the development of treatments that may be used as prevention, avoiding the progression of the attack and the onset of the headache phase. Moreover, both stress and kappa activation led to increased circulating levels of prolactin (PRL) in the blood, both in humans (Bernard, Young, and Binart 2019; Kreek et al. 1999; Lennartsson and Jonsdottir 2011; Levine and Muneyyirci-Delale 2018) and rodents (Guerrero et al. 2019; Bryant et al. 1998; Butelman and Kreek 2001; Zullig et al. 2007), which is associated with increased headaches in people who suffer from migraine (Bosco et al. 2008; Kallestrup et al. 2014). In addition, patients with prolactinoma-associated hyperprolactinemia show an increased incidence of migraine. PRL has been also found to co-localize with $5HT_{1B}$ or $5HT_{1D}$ receptors in the trigeminal nociceptors and has been shown to selectively sensitize nociceptors in female mice more than in males. Watanabe and colleagues (Watanabe et al. 2022) demonstrated the hypothesis

that repetitive stress could involve the hypothalamic kappa receptor with a consequent increase of the PRL levels and dysregulation of the PRL receptors at the level of trigeminal neurons of female mice, causing the onset of peripheral allodynia. They demonstrated how the deletion, or the blockade, of the kappa receptor, prevents the increase of PRL after repeated stress sessions and the consequent cutaneous allodynia in female mice. Aticaprant is a kappa opioid antagonist now in clinic phase III (ClinicalTrials.gov Identifier: NCT05455684) as an antidepressant drug. Considering the promising results with the pre-clinical models of stress-induced migraine, this compound may be tested in the near future for its ability to prevent migraine in stress susceptible patients.

Delta-opioid receptor – delta receptors are expressed in several regions involved in migraine such as trigeminal and dorsal root ganglia, in which it is co-express with CGRP (Bardoni et al. 2014), in trigeminal nucleus caudalis, and cortex (Pradhan et al. 2011; Mansour et al. 1988; Mennicken et al. 2003). Other sites of expression are striatum, hippocampus and amygdala (Pradhan et al. 2011; Lutz and Kieffer 2013). If compared to mu-opioid receptor agonists, delta agonists have a lower abuse liability and do not cause rewarding behaviors or physical dependence in non-human primates and rodents (Negus et al. 1998; Brandt et al. 2001). Moreover, delta agonists produce fewer respiratory depression and fewer side effects on gastrointestinal transit than mu agonists (May et al. 1989; Gallantine and Meert 2005). Despite all these advantages, delta agonists show a tendency to produce proconvulsant effects (Pradhan et al. 2012), and they are not highly effective in acute pain (Vicente-Sanchez, Segura, and Pradhan 2016), but show good efficacy in a chronic pain state such as inflammatory or neuropathic pain (Vicente-Sanchez, Segura, and Pradhan 2016). Some studies reported the ability of delta agonists to counteract headache in different animal models. In 2014 Pradhan and colleagues reported the effectiveness of the delta agonist SNC80, ARM390, and JNJ20788560 in reducing GTN-induced allodynia and place aversion and in reducing KCL-induced CDS (Pradhan et al. 2014). Similar results were reported by Dipps et al. (Dripps et al. 2018). Subsequently, Moye et al. reported SNC80 being effective in a post-traumatic headache model (Moye, Novack, et al. 2019) and in MOH induced by sumatriptan and morphine (Moye, Tipton, et al. 2019). Recently an increase in delta expression has been reported in the cortex, hippocampus, and striatum after GTN administration and the delta receptor express in these area have reveled necessary for the anti-migraine effects of SNC80 (Dripps et al. 2020). All these studies suggest the potential therapeutic of delta agonists as anti-migraine drugs, both for acute and preventive therapies. To this regards, it is worth of mention that in mice the chronic administration of a

delta agonist produced only slight MOH, compared to sumatriptan and morphine (Moye, Tipton, et al. 2019).

Mu-opioid receptor – mu agonists such as morphine, hydrocodone, and oxycodone are prescribed when other classes of drugs failed to revert the migraine state (Marcelo E Bigal and Lipton 2009). Despite their effectiveness, their use lead to several side effects such as MOH, chronification of the headaches (Marcelo E Bigal and Lipton 2009), tolerance and possibly dependence depression crises, and gastrointestinal motility disturbances, whit a high impact on public health costs (Colás et al. 2004; Reid et al. 2002; H.-C. Diener et al. 2016). In order to avoid these side effects, several strategies have been developed, one of these is the development of mu agonists with a functional selectivity, strongly selective for the G-protein pathway (biased agonists). One of these molecules, Oliceridine, has been approved in 2020 by FDA for the use in the treatment of acute pain of sufficient severity to require an intravenous opioid analgesic for which alternative treatments are inadequate (Azzam and Lambert 2022).

Another strategy is the development of "mixed opioid" molecules, because of the preclinical evidence that mu/delta mixed antagonist might offer antinociceptive advantage (Dietis et al. 2009). Also, the NOP/OP mixed agonists seem to be effective, indeed the mixed opioid-NOP ligand Cebranopadol is in advanced clinical trials (Calo and Lambert 2018).

1.6.2 The nociception orphanin/FQ receptor

The nociceptin/orphanin FQ (N/OFQ) peptide (NOP) receptor, discovered in 1994, is a G-coupled receptor with high homology to the classical opioid receptors (Mollereau et al. 1994), and because of this, the NOP receptor is considered the fourth member of the opioid-receptor family. Subsequently, two groups of researchers discovered the selective ligand of this receptor, an endogenous 17-amino peptide (Meunier et al. 1995; Reinscheid et al. 1995). NOP receptor is a G_i-coupled receptor and its activation led to the activation of G protein Inwardly Rectifying Potassium (GIRK) channels and consequently cells hyperpolarization that causes a reduced cellular activation (Chiou et al. 2004). The N/OFQ prepropeptide and its receptor (NOP) are expressed in the central and peripheral nervous systems. The activation of the NOP receptor by N/OFQ modulates several biological functions such as pain transmission, locomotor activity, stress and anxiety, emotional states, learning and memory, and others (Lambert 2008).

Several evidence link the N/OFQ-NOP receptor system to migraine pathophysiology.

The NOP receptor and N/OFQ have been found highly expressed in migraine-associated brain structures such as the trigeminal ganglia and trigeminal nucleus caudalis both in mice and rats. In mice the NOP receptor co-localized with CGRP, substance P, and nitric oxide synthase (Targowska-Duda et al. 2020; Ozawa et al. 2015; Neal, Akil, and Watson 2001; Kiguchi et al. 2020; Hou et al. 2003). The NOP receptor is expressed also in human trigeminal ganglia (Witta et al. 2004; Hou et al. 2003; G. X. Xie et al. 1999; Mørk et al. 2002). In an in vitro study done on primary cultures of rat trigeminal ganglion neurons, N/OFQ inhibited calcium channel current voltage-dependent, showing an inhibitory effect on neuronal activity (Borgland, Connor, and Christie 2001). In addition, the N/OFQ analog, N/OFQ(1-13)NH_2, was found to inhibit the release of CGRP induced by various stimuli such as capsaicin, veratridine, and potassium chloride in primary cultures of trigeminal ganglion neurons. The inhibitory effect of N/OFQ(1-13)NH_2 is selectively mediated by the activation of the NOP receptor, as it is completely reverted by the selective antagonist [N(Phe^1)]N/OFQ(1-13)NH_2 (A Capuano et al. 2007). Using the same biological preparation, further studies have been conducted to assess the involvement of the NOP receptor in CGRP release modulation. These have shown that buprenorphine is able to block the secretion of CGRP induced by bradykinin and this effect is blocked by both naloxone and the NOP antagonist [N(Phe^1)]N/OFQ(1-13)NH_2. This confirms that activation of the NOP receptor at the level of the trigeminal ganglion inhibits the release of CGRP (Alessandro Capuano et al. 2009). Again, the NOP receptor's pharmacological modulation was observed to regulate the neurogenic dural vessel vasodilatation in rats (Bartsch,

Akerman, and Goadsby 2002). Given these pieces of evidence, it could be hypothesized that the inhibitory effect of N/OFQ in dural vasodilation is mediated by the suppression of the release of CGRP from the trigeminal nerves that innervate the meninges. Electrophysiology studies in the anesthetized rat have shown inhibitory N/OFQ activity on neurons of the trigeminal nucleus caudalis. In particular, applied locally, N/OFQ inhibits excitatory responses evoked by the excitatory amino acids N-methyl-D-aspartate (NMDA) and α-amino 3-hydroxy 5-methyl 4-isoxazolpropionic (AMPA) (X. M. Wang, Zhang, and Mokha 1996). A few years later a study confirmed the inhibitory effect of N/OFQ on the excitatory action of NMDA in male rats but not in females, suggesting that sex and sex hormone levels may affect N/OFQ inhibitory modulation (Flores et al. 2001). Some behavioral studies have been also carried out. It has been shown that the non-peptide NOP agonist, Ro 65-6570 (0.1-1mg/kg, intraperitoneal (i.p.)), produces a robust dose-dependent analgesic effect in the orofacial formalin test (A Rizzi et al. 2017). Similar effects were obtained with the NOP/opioid agonist cebranopadol (A Rizzi et al. 2017). Ro 65-6570 was able also to reduce the mechanical allodynia induced by the injection of GTN in the study by Targowska-Duda and colleagues. These analgesic effects are blocked by the selective NOP antagonist SB-612111.

These evidence suggest the NOP receptor a promising pharmacological target for the development of new migraine treatment, worth to be further investigated and validated.

NOP/opioid mixed agonists – different complimentary findings suggest that coactivation of the NOP and the mu opioid receptors can represent an interesting pharmacological approach for the development of innovative anti-migraine drugs. In fact the simultaneous NOP/mu activation is reported to elicit synergistic antinociceptive effects with a better tolerability profile than those of the classical opioid analgesics acting as pure mu agonists (Figure 14).

Some NOP/mu mixed agonists now exist. The main exponent of this class is Cebranopadol. Cebranopadol is a mixed NOP/opioids receptor agonist developed by Grunenthal and now in clinical phase III. Cebranopadol is a mixed NOP and opioid agonist, with high potency and efficacy at the NOP and mu receptors (Anna Rizzi et al. 2016; Linz et al. 2014). In several behavioral studies conducted in rodents using different pain models (nociceptive, neuropathic and inflammatory pain, arthritic pain, chemotherapy-induced neuropathic pain) Cebranopadol elicits potent antinociceptive effects (Schunk et al. 2014; Schiene et al. 2018; Anna Rizzi et al. 2016; Linz et al. 2014). Importantly, both naloxone and NOP selective antagonists (J-113397 and SB-612111) were able to prevent Cebranopadol analgesic effect, demonstrating that the concomitant activation of mu and NOP recptors is required for the

antinociceptive action of this compound (Schiene et al. 2018; Anna Rizzi et al. 2016). Interesting enough, Cebranopadol was able to counteract mouse nociceptive behaviors in a model of inflammatory pain in the trigeminal territory, suggesting the utility of this compound for the migraine treatment (A Rizzi et al. 2017). Preclinical studies demonstrated the safer profile of Cebranopadol compared to those of the classical mu agonists, Specifically, the administration of Cebranopadol in rodents does not influence motor coordination at the rotarod test (Linz et al. 2014; Anna Rizzi et al. 2016); Cebranopadol induced lower respiratory depression than fentanyl in rats (Linz et al. 2014); Cebranopadol displayed low liability to induce tolerance (Linz et al. 2014) and physical dependance than mu agonist (Tzschentke et al. 2019; Ruzza et al. 2019). Antagonism and knockout experiments demonstrated that is the NOP activation that prevents mu-dependent respiratory depression and physical dependance, making the therapeutic index larger for Cebranopadol than for classical mu agonists (Linz et al. 2014; Ruzza et al. 2019). Cebranopadol entered several clinical trials to test its tolerability and efficacy in patients that suffer from acute and chronic pain [ClinicalTrials.gov Identifier: NCT00872885; NCT01939366; NCT01357837; NCT01709214; NCT01725087; NCT01347671; NCT00878293]. For a review of these studies, please see (Calo and Lambert 2018). Currently, data from clinical trials demonstrated Cebranobadol a safe and effective analgesic. Over the years, other mixed NOP/mu agonists have been synthetized and pharmacological characterized in pre-clinical models. AT-121 and BU10038 rapresent two exemple of such compounds. AT-121 is a compound that acts as a mixed NOP/mu partial agonist with high binding affinity (Ding et al. 2018). In monkeys, its subcutaneous administration produced morphine-like analgesic and antiallodynic effects, without triggering respiratory depression and with low tolerance and abuse liability. Similarly, BU10038 is a naltrexone-derived mixed NOP/mu partial agonist synthesized by Husbands and Ko groups (Kiguchi et al. 2019). Its systemic and intrathecal administrations in non-human primates, resulted in a long-lasting antinociceptive effect with neither reinforcing effects, nor other effects like itching, respiratory depression, and tolerance when administered repeatedly. In conclusion, the large amount of scientific literature produced with Cebranopadol and some works performed with different compounds, strongly suggest that mixed NOP/opioid ligands as promising, highly effective, and safe pain treatments. Considering the evidence linking migraine and NOP receptor and the fact that mu agonists are already used in the clinic to treat migraine when other drugs are not enough, we hypothesize that mixed NOP/mu or NOP/opioid agonists are worthy of being investigating as new antimigraine drugs.

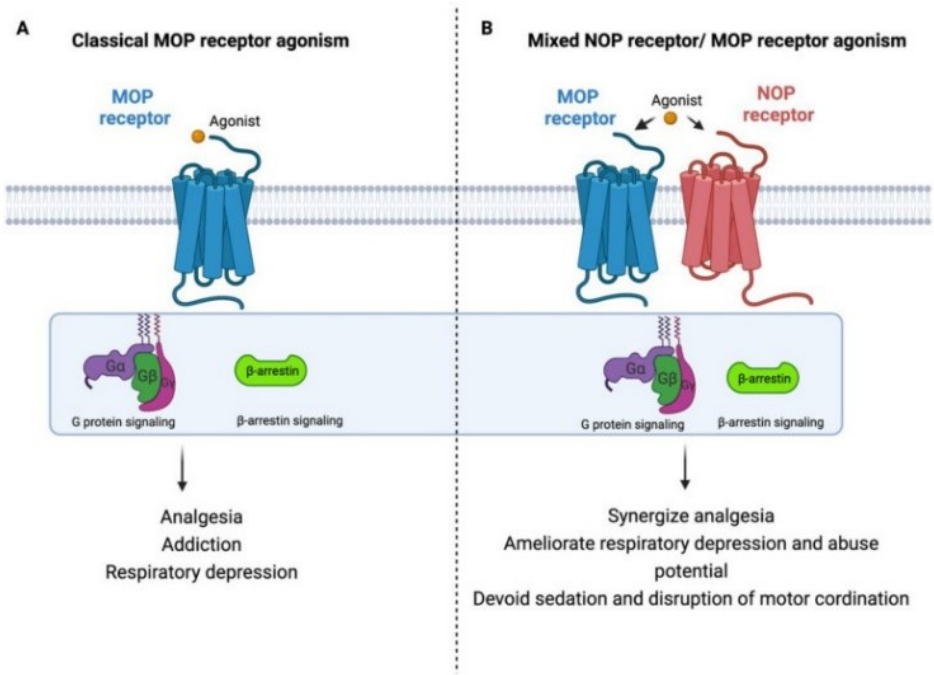

Figure 14. Figure taken from (El Daibani and Che 2022). (**A**) Beneficiary and side effects produced by MOR activation. (**B**) Synergizing analgesic effect and protective effects produced by mixed compound with simultaneous agonistic activity at NOP receptor and MOR receptor.

1.6.3 TRPA1 receptor

The transient receptor potential (TRP) channel superfamily is the most represented family of receptors on primary sensitive terminations (Nilius and Szallasi 2014). It is made up of over 50 heterogeneous members who share the role of sensory transducers. This family is composed of 6 subfamilies and 28 members acting as ion channels that allow the passage of cations including calcium, sodium, and potassium, with consequent depolarization of membranes, activation of the action potential, and transduction of the nociceptive signal (Iannone et al. 2022). TRP receptors consist of six transmembrane domain (called S1-S6 or TM1-TM6) proteins assembling as homo- or hetero-tetramers. Between the subunits S5 and S6, there is a conductive ion pore. Both the aminic (NH_2) and carboxylic (COOH) ends are localized at the cytosolic level. If the COOH part is highly conserved within the different families, the NH_2 has characteristic differences in each receptor subtype (González-Ramírez et al. 2017). The six subfamilies are divided into TRP canonical (TRPC), TRP vanilloid (TRPV), TRP mucolipin (TRPML), TRP melastatin (TRPM), TRP polycystin (TRPP), and TRP ankyrin (TRPA). All these subfamilies include several members, whit the exception of the TRPA subfamily, with only one member: the TRPA1. These receptors are activated by a wide range of exogenous stimuli including mechanical stimuli, temperature, pH, and also endogenous stimuli like intracellular mediators (i.e. various phospholipase C isoforms). Some members of the TRP family are widely expressed in the subpopulation of primary sensory neurons where they are involved in the detection of chemical and physical nociceptive stimuli (Hung and Tan 2018). Some of these channels are TRPV1 and TRPA1. It is worth noting that those channels are strongly involved inflammatory and neuropathic pain transmission, and they are co-localized at the level of primary sensory neurons of the dorsal root ganglion (DRG), trigeminal ganglion (TG), and vagal ganglia (VG), especially at the level of the nociceptive nerve fibers of type C and Aδ, in peptidergic and non-peptidergic neurons. TRPA1 has been detected also in the central nervous system (CNS) (i.e. cortex, caudate nucleus), as well as in non-neuronal cells like Schwann cells, vascular endothelial cells, skin fibroblasts, and pulmonary epithelium (De Logu et al. 2017; Iannone et al. 2022). At the level of peptidergic nociceptors, the stimulation of TRPV1 and TRPA1, activated by noxious stimuli or by selective agonists, causes the release of some neuropeptides such as the substance P (SP), and the calcitonin gene-related peptide (CGRP), two main mediators of neurogenic inflammation, that are responsible for plasma protein extravasation, and arteriolar vasodilation (De Logu, Nassini, et al. 2019).

TRPA1 is a sensor of a wide variety of harmful external disturbances such as intense cold, pungent compounds (like mustard oil, allicin, and isothiocyanates), environmental irritants,

and components of cigarette smoke (i.e. acrolein, crotonaldehyde, nicotine, formaldehyde) (De Logu, Landini, et al. 2019; Dussor et al. 2014) and is also activated by a wide variety of endogenous products released in inflammatory conditions, especially the products of oxidative stress, like reactive oxygen (ROS), nitrogen (RNS), and carbonyl species (RCS) like 4-hydroxynoneal (4-HNE) (Takahashi and Mori 2011). Considering the type of signaling and its location, TRPA1 gets attention as a major pain transducer. In fact, the calcium influx induced by ROS, RNS, and RCS is blocked by TRPA1 antagonists and is not present in TRPA1 knockout mice. In particular, peripheral nerve injury-induced allodynia is reduced after TRPA1 blockage, as well as hypersensitivity following injury in a spinal nerve ligature model (Wei et al. 2011). Again, TRPA1 expressed in Schwann cells was involved in the mechanical allodynia caused in mice by a partial sciatic nerve ligature (De Logu et al. 2017), alcoholic neuropathy (De Logu, Li Puma, et al. 2019), and cancer (De Logu et al. 2021).

There is also evidence of the involvement of TRPA1 in migraine, in fact, it has been shown as TRPA1 induces vasodilation at the meningeal level following activation by some environmental irritants known to provoke migraines in humans, such as cigarette smoke and formaldehyde (Edelmayer et al. 2012). Additionally, TRPA1 activation was demonstrated to be required for the development of allodynia in different migraine models. It was for example demonstrated that Umbellulone, a substance present in the leaves of Umbellularia californica (the ‘headache tree’) is able to induce migraine through the TRPA1 activation at the level of the trigeminovascular system (Nassini et al. 2012). Also, the subcutaneous injection at the level of the periorbital area, such as the dural stimulation, of allyl isothiocyanate (AITC) elicits a proalgesic response in the Von Frey test (Marone et al. 2018; Burgos-Vega et al. 2019) . Again, TRPA1 polymorphism seems to be linked to migraine generation (Nilius and Szallasi 2014). Moreover, it was demonstrated that common drugs used to treat migraines such as acetaminophen, via its reactive metabolite NABQI (Andersson et al. 2011; Nassini et al. 2010), and dipyrone act, at least in part, by blocking TRPA1 (Nassini et al. 2015). Finally, TRPA1 activation have been reported necessary in mice for GTN induced allodynia it has been demonstrated that nitric oxide produced by GTN aldehyde dehydrogenase 2, is necessary to start the whole process, but it is not enough for maintenance of migraine status in mice. Allodynia in this model depends by the activation of TRPA1 in the trigeminal nociceptor and the subsequent release of ROS and CGRP. It is interesting to note that allodynia, but not vasodilatation, is prevented by gene deletion or pharmacological blocking of TRPA1 (Marone et al. 2018).

All these findings together with its expression at the level of the nociceptive nerve fibers, make TRPA1 promising for several painful pathologies, including migraine. Specifically, the blockage of TRPA1 may represent an effective new pharmacological approach.

1.6.4 Cannabinoid

Cannabidiol (CBD) is the main non-psychotropic component present in the *Cannabis Sativa*, with therapeutical potential over a wide range of disorders that result from an equally wide range of CBD's pharmacological actions. Early reports indicate that *Cannabis* was used quite extensively as an effective prophylactic and abortive treatment for migraine and other headache disorders (Lochte et al. 2017). However, the schedule 1 classification of marijuana in 1970 in the United States has challenged the clinical evaluation of this substance and its components with respect to headache disorders. Except for a few clinical studies where the combination of Δ^9-THC and CBD was tested (Lochte et al. 2017; Rhyne et al. 2016), there is no available information about the clinical use of CBD as a migraine treatment (Lochte et al. 2017; Rhyne et al. 2016). Despite these limitations, evidence suggests an analgesic effect of CBD due to its interaction with TRPV1 (De Gregorio et al. 2019; Costa, Giagnoni, et al. 2004), an anti-inflammatory effect (Costa, Colleoni, et al. 2004), a therapeutic effect in various pain-related conditions including anxiety (De Gregorio et al. 2019), and the ability to modulate of the serotoninergic system (De Gregorio et al. 2019), which is widely involved in trigeminal activation (Hoskin, Kaube, and Goadsby 1996), a common mechanism for migraine pain. Consistent with all these pharmacological actions, the pharmacodynamics of CBD is complicated. CBD competes poorly with cannabinoid ligands at the level of cannabinoid receptors (Thomas et al. 1998), suggesting low affinity for these receptors. In addition to interacting with the cannabinoid system, CBD acts on several other targets, including various receptors, enzymes, and ion channels (Tham et al. 2019). Currently, it is been proposed that CBD's effect is mediated by the serotoninergic GPCR 5-HT_{1A} receptor via positive allosteric modulation (E. B. Russo et al. 2005). Also, other orphan CGPRs linked with cytoskeletal remodeling, plasma membrane topology, neurite outgrowth, cell survival, and proliferation have been modulated by CBD (Ryberg et al. 2007; Laun et al. 2019). Other studies suggested the binding with mu and delta opioid receptors (Bian et al. 2019). Moreover, CBD binds several TRP channels, including TRPA1, TRPV1 and TRPV4 (De Petrocellis et al. 2011, 2008; Bisogno et al. 2001). Furthermore, the nuclear receptor peroxisome proliferator-activated receptor gamma (PPARγ) is targeted by CBD that, alongside the modulation of expression patterns in microglia through the activation of nuclear factor erythroid-derived 2-like 2 (Nrf2), proposes this molecule as an important clue in the inflammation pathway modulation (Granja et al. 2012; Juknat et al. 2013). Several studies demonstrated the analgesic potential of CBD in several pain-related diseases.

The action of CBD in neuropathic pain models – CBD has a pain-relieving effect on several models of neuropathic pain. In a model of neuropathic pain induced by the ligation of the sciatic nerve in mice, CBD administered orally significantly reduced allodynia up to 3 weeks post-surgery, similar to Δ^9-THC and morphine. Both CBD and Δ^9-THC did not elicit tolerance at the extent of morphine (Abraham et al. 2020). In the L5 spinal nerve ligation model in rats, CBD exerts its analgesic effects on chronic neuropathic pain due to potentiation of the α3 glycine receptor, and true CB1- and/or CB2 receptor-mediated mechanisms (Xiong et al. 2012). In a mouse neuropathic pain model involving foramen rotundum inflammatory constriction trigeminal infraorbital nerve injury, orally administered CBD reduced mechanical allodynia after 1 hour from the administration (Vigil et al. 2020). Its analgesic effect was also studied in a chronic constriction injury model of neuropathic pain, through both the oral and subcutaneous administration. CBD was able to reduce mechanical and thermal allodynia (Casey, Atwal, and Vaughan 2017; Costa et al. 2007). Lie et al. also demonstrated that CBD acts as an anti-inflammatory agent through the inhibition of the extravasation of cytokine and chemokine after spinal cord injury (Li et al. 2018). CBD was able to modulate serotoninergic transmission at the level of the dorsal raphe nucleus. In a model of spared nerve injury in rats, CBD decreased mechanical allodynia, and anxiety-like behavior and normalized the serotoninergic activity (De Gregorio et al. 2019). CBD also blocked chronic pain triggered by the administration of paclitaxel (PAC), an effect reverted by the 5-HT_{1A} receptor antagonist WAY100635 (Ward et al. 2011, 2014; King et al. 2017). Additionally, CBD reduced neuropathic pain induced by cisplatin in mice and prevented oxidative stress, inflammation, and cell death in the kidney (Pan et al. 2009) but did not prevent pain development when given 30 minutes prior to cisplatin (Harris et al. 2016).

Neuropathic pain is often associated with type 1 diabetes. In a rodent model of diabetes pain, CBD elicited a positive action by delaying the development of the disease, ameliorating the pancreatic microcirculation, reducing oxidative stress, cardiac fibrosis, inflammation and cell death, while improving the ability of arteries to relax thanks to better production of products derived from vasodilative cyclooxygenase (Wheal et al. 2014; Lehmann et al. 2016; Rajesh et al. 2010). Moreover, in diabetic rats, acute CBD produced an antiallodynic effect in the Von Frey test through a serotonin-mediated pathway (Jesus et al. 2019).

CBD in inflammatory pain – Cannabinoids have been reported to have analgesic and anti-inflammatory effects in studies that used inflammatory-induced chronic pain models. CBD and its modified derivatives ameliorated the condition of complete Freund's adjuvant (CFA)-

induced inflammatory pain rodent model (Xiong et al. 2012). In the topic application of 2.5% Croton oil in acetone and i.p. administration of lipopolysaccharide (LPS), CBD inhibited the release of pro-inflammatory factors such as interleukin 6 (IL-6) and tumor necrosis factor (TNF) and increased the levels of the anti-inflammatory cytokine interleukin 10 (IL-10) (Verrico et al. 2020). Another study reported a significant reduction in IL-1b, IL-10, and interferon γ (IFN- γ) levels and an increase in IL-6 levels in the CFA-induced inflammatory pain model (Britch et al. 2020). Lastly, experimental autoimmune encephalomyelitis in mice was ameliorated by CBD administration, with a reduction in axonal damage, inflammation, and T-cell recruitment in mice spinal cord (Kozela et al. 2011).

Clinical studies – Only 1 CBD product has been approved by FDA for the clinical usage: Epidiolex is a pure CBD formulation used clinically to reduce seizures in Lennox Gastaut syndrome, and Dravet syndrome in patients > two years old. Anyway, several clinical studies have been performed to test the utility of CBD in different conditions, i.e. epilepsy, anxiety, pain/inflammation, schizophrenia, various substance use disorders, and post-traumatic stress disorder (for a review in this field see (Sholler, Schoene, and Spindle 2020)). Regarding pain, only a few clinical studies have evaluated the efficacy of CBD alone. Almost all studies conducted in this area have coadministered CBD with THC. Co-administration of CBD with THC reduced pain improves sleep quality by reducing insomnia and fatigue (Hoggart et al. 2015; Johnson et al. 2013). Sativex contains both CBD and THC and clinical studies have shown its effectiveness in pain reduction in various pathologies such as multiple sclerosis, cancer or rheumatoid arthritis (M. Russo et al. 2016; Portenoy et al. 2012; Nurmikko et al. 2007; Blake et al. 2006). Taken together, these initial findings imply that CBD represents a promising phytocannabinoid-based treatment option in the treatment of pain conditions, including migraine.

1.7 Aim of the study

A primary focus of migraine research is the identification of the mechanistic basis of the disease. Addressing the need to discover effective antimigraine drugs or preventive treatments is a direct implication of this research focus. Thanks to the extensive research work done in the last decades, the knowledge about migraine pathophysiology has grown, and new possible pharmacological targets to counteract the disease have been identified. The general aim of my research work has been the in vitro or the in vivo pharmacological investigation of new and standard compounds acting on different receptors, known as players in migraine disease. In particular, 5 specific aims (SA) have been developed:

1. Set-up and pharmacological validation of a battery of in vitro assays to characterize ligands for the kappa opioid receptors. Specifically, the following assays have been used: calcium mobilization assay; dynamic mass redistribution (DMR); iii) bioluminescence resonance energy transfer assay (BRET) to evaluate kappa receptor interaction with G protein and β-arrestin 2. In the frame of this study the two new dynorphine A derivatives have been characterized: PWT2-Dyn A and Dyn A-palmitic.
2. In vitro pharmacological characterization of new mixed NOP/mu peptide agonists. Specifically 31 new compounds with the general sequence [Tyr/Dmt^{1}Xaa^{5}]N/OFQ(1-13)NH2 have been synthetized in Prof. Guerrini's (University of Ferrara) laboratories and pharmacologically investigated in our laboratories using the calcium mobilization and the DMR assay and cells stably expressing the NOP, mu, delta and kappa receptors.
3. In vitro pharmacological characterization of new TRPA1 antagonists. These compounds have been synthetized in Prof. Preti's (University of Ferrara) laboratories and pharmacologically investigated in our laboratories using the calcium mobilization. All the compounds were analogues of the standard TRPA1 antagonist DHC200.
4. In vivo evaluation of CBD in a mouse model of migraine induced by CGRP. Specifically, CBD has been evaluated for its ability to prevent and block acute migraine attack and for its ability to prevent the development of chronic migraine.
5. Investigation of the involvement of the NOP receptor in migraine by studying the phenotype of mice knockout for the NOP receptor in two experimental migraine models (NTG induced migraine and CGRP induced migraine).

2. RESEARCH WORK

2.1 Pharmacology of Kappa Opioid Receptors: Novel Assays and Ligands

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Pharmacology of Kappa Opioid Receptors: Novel Assays and Ligands

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The present study investigated the *in vitro* pharmacology of the human kappa opioid receptor using multiple assays, including calcium mobilization in cells expressing chimeric G proteins, the dynamic mass redistribution (DMR) label-free assay, and a bioluminescence resonance energy transfer (BRET) assay that allows measurement of receptor interaction with G protein and β-arrestin 2. In all assays, dynorphin A, U-69,593, and [D-Pro^{10}]dyn(1-11)-NH$_2$ behaved as full agonists with the following rank order of potency [D-Pro^{10}]dyn(1-11)-NH$_2$ > dynorphin A ≥ U-69,593. [Dmt^{1},Tic^{2}]dyn(1-11)-NH$_2$ behaved as a moderate potency pure antagonist in the kappa-β-arrestin 2 interaction assay and as low efficacy partial agonist in the other assays. Norbinaltorphimine acted as a highly potent and pure antagonist in all assays except kappa-G protein interaction, where it displayed efficacy as an inverse agonist. The pharmacological actions of novel kappa ligands, namely the dynorphin A tetrameric derivative PWT2-Dyn A and the palmitoylated derivative Dyn A-palmitic, were also investigated. PWT2-Dyn A and Dyn A-palmitic mimicked dynorphin A effects in all assays showing similar maximal effects but 3–10 fold lower potency. In conclusion, in the present study, multiple *in vitro* assays for the kappa receptor have been set up and pharmacologically validated. In addition, PWT2-Dyn A and Dyn A-palmitic were characterized as potent full agonists; these compounds are worthy of further investigation *in vivo* for those conditions in which the activation of the kappa opioid receptor elicits beneficial effects e.g. pain and pruritus.

Keywords: kappa opioid receptor, G protein-coupled receptor, label-free, BRET, calcium mobilization, biased agonism, PWT2-dyn A, dyn A-palmitic

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INTRODUCTION

The kappa opioid receptor was cloned in 1993 (Meng et al., 1993), while its pharmacological identification spans more than two-decades before. Portoghese and others proposed more than a single opioid receptor type existed (Portoghese, 1965). The earliest direct demonstrations of multiple opioid receptor binding sites were obtained with radiolabelled naloxone and etorphine molecules (Pert and Snyder, 1973; Simon et al., 1973; Terenius, 1973). The first definitive evidence that these receptors did not form a homogeneous population was presented in 1976 (Martin et al., 1976), and kappa opioid receptors as specific subtype confirmed with structure-activity relationship studies on the dynorphin(1-13) scaffold (Chavkin and Goldstein, 1981). Subsequent medicinal chemistry and

molecular biology studies identified major ligand binding and receptor domains (Hjorth et al., 1995; Hjorth et al., 1996; Thirstrup et al., 1996; Cheng et al., 1998; Coward et al., 1998; Jones et al., 1998; Bonner et al., 2000; Celver et al., 2002; Owens and Akil, 2002; Zhang et al., 2002; Pascal and Milligan, 2005; Yan et al., 2005): discoveries recently confirmed via X-ray crystallography (Wu et al., 2012; Che et al., 2020).

Kappa opioid receptor is a G protein-coupled receptor (GPCR), whose activation mediates guanosine diphosphate (GDP) to guanosine triphosphate (GTP) exchange in the alpha subunit of pertussis toxin-sensitive heterotrimeric G proteins. These G proteins dissociate and activate downstream cascades, including depression of cyclic adenosine monophosphate (cAMP) formation, opening of potassium and closing of voltage-gated calcium channels. Activated kappa opioid receptors interact with G protein-coupled receptor kinases (GRK: GRK2,3,5,6, (Chen et al., 2016; Chiu et al., 2017)) leading to C-terminal phosphorylation; this allows β-arrestin interaction.

Kappa opioid receptor agonists were identified to differentially trigger G protein or β-arrestin coupling (Mores et al., 2019; Faouzi et al., 2020); this capacity, i.e. ligand-dependent activation of some but not other specific transducer of a given GPCR, is called biased agonism (Kenakin, 2017). Very intriguingly, efforts have been made to model biased agonists activity and opioid receptors domain binding: G protein bias involves transmembrane domain V and extracellular loop II, while β-arrestin bias transmembrane domains II and III (Uprety et al., 2021). Moreover, several ligands were studied for their coupling preferences towards the different Gi/o (Olsen et al., 2020). The reason for this focus on the different transductional fingerprints of the kappa opioid receptor lies in the possibility to discriminate its *in vivo* effects i.e. therapeutic vs side-effects. The kappa opioid receptor is a key player in pain and mood modulation. Kappa opioid receptor agonists display a potent antinociceptive effect; however, their clinical use has been strongly limited because dysphoria, hallucinations, and dissociation accompany therapeutic effects. For the kappa opioid receptor, G protein engagement over β-arrestins interaction has been proposed as the preferred profile (Bruchas and Chavkin, 2010). Importantly, therapeutic advantage by kappa opioid receptor activation might be obtained through the development of 1) G protein-biased, 2) peripherally restricted, and 3) mixed opioid agonists (Paton et al., 2020). Intriguingly, nalfurafine is marketed (in Japan) as an antipruritic drug. It is a selective kappa opioid agonist (Inui, 2015), reportedly biased towards G protein (Mores et al., 2019), particularly at human compared to rodent receptors (Schattauer et al., 2017), and it is currently the sole marketed selective kappa opioid receptor agonist. Captivating insights come from novel omics approaches are modifying the scenario of GPCR biased agonism by studying several (virtually all) possible transduction mechanism at a time. Such studies linked the mammalian Target of Rapamycin pathway to the aversive behavior of kappa opioid receptor agonists (Liu et al., 2019).

Blocking the kappa opioid receptor is recommended to treat depressive, anxiety, and substance use disorders (Lutz and Kieffer, 2013; Carlezon and Krystal, 2016). GPCRs residence time (reciprocal of the dissociation rate constant) has been described as good predictor of *in vivo* efficacy (Strasser et al., 2017; van der Velden et al., 2020). However, as stated by Page and co-workers the peculiarly slow pharmacodynamic of kappa opioid receptor antagonists complicate their early phase clinical investigation; therefore, the development of shorter-acting antagonists is needed and might provide insight into whether these drugs are efficacious as predicted in preclinical studies (Carroll and Carlezon, 2013; Page et al., 2019).

In this study we deployed multiple assays to study the pharmacology of standard kappa opioid receptor ligands with differing pharmacological properties then to characterize and compare two novel dynorphin A (Dyn A) derivatives: PWT2-Dyn A and Dyn A-palmitic. PWT2-Dyn A is a tetrabranched derivative obtained by jointing four molecules of [Cys^{18}]Dyn A to a PWT2 core. Dyn A-palmitic was obtained by reaction of [Cys^{18}] Dyn A with a palmitoyl functionalized maleimide moiety. Specifically, we report data using calcium mobilization, dynamic mass redistribution (DMR), and bioluminescence resonance energy transfer (BRET) receptor-transducer interaction assays.

METHODS

Drugs

Peptides Dyn A, [D-Pro^{10}]Dyn(1-11)-NH_2 ([D-Pro^{10}]) and [Dmt^1,Tic^2]Dyn(1-11)-NH_2 ([Dmt^1,Tic^2]) were synthesized in line with solid phase peptide synthesis methodology previously reported (Gairin et al., 1986; Guerrini et al., 1998). The homo tetrameric PWT2-Dyn A was obtained grafting four molecules of [Cys^{18}]Dyn A with the PWT2 core in a classical thiol-Michael reaction using experimental conditions previously optimized for the synthesis of nociceptin/orphanin FQ tetra branched derivatives (Guerrini et al., 2014). Similarly, the Dyn A analogue, Dyn A-palmitic was synthesized reacting in solution [Cys^{18}]Dyn A with a palmitoyl functionalized maleimide moiety (Pacifico et al., 2020).

The non-peptide molecules U-69,593, Nor-Binaltorphimine (Nor-BNI), and GDP were from Tocris bioscience (Bristol, United Kingdom). All tissues culture media and supplements were from Invitrogen (Paisley, United Kingdom). Reagents used were from Sigma Chemical Co. (Poole, United Kingdom) and were of the highest purity available.

Concentrated solutions of ligands were made in ultrapure water (1 mM, peptides; 10 mM, GDP) or dimethyl sulfoxide (10 mM) and kept at - 20°C until use.

Calcium Mobilization Assay

Chinese Hamster Ovary (CHO) cells stably co-expressing the human kappa or mu opioid receptors and the $G\alpha_{qi5}$ protein or the human delta and the $G\alpha_{qG66Di5}$ protein were used in this assay. Cells were generated as described by Camarda and co-workers (Camarda and Calo, 2013). They were cultured in

DMEM/F-12 (1:1) medium supplemented with 10% FBS, 2 mM L-glutamine, 200 mg/ml G418, 100 IU/ml penicillin, 100 IU/ml streptomycin, and 1 µg/ml fungizone. In the assays, cells were maintained at 37°C in a humidified atmosphere with 5% CO_2. After reaching confluence, cells were detached by trypsinization, and 50,000 cells/well were seeded into 96-well black, clear-bottom plates 24 h before the test. At the assay time, cells were pre-incubated for 30 min at 37°C protected from light with a loading solution consisting of HBSS supplemented with 2.5 mM probenecid, 3 µM Fluo-4 AM, and 0.01% pluronic acid. The loading solution was subsequently discarded, and 100 µL/well of assay buffer consisting of HBSS with 20 mM HEPES, 2.5 mM probenecid, and 500 µM Brilliant Black (Sigma-Aldrich, St. Louis, United States) was dispensed and incubated for an additional 10 min under the same conditions. Serial dilutions of ligands were prepared in HBSS buffer with 20 mM HEPES and 0.02% of bovine serum albumin (BSA) to minimize ligands' stickiness to plasticware. The automated microplate reader FlexStation II (Molecular Device, Union City, CA 94587, United States) was employed to detect changes in fluorescence intensity. Experiments were carried out at 37°C. Automated additions were carried out in a volume of 50 µL/well. In antagonism experiments, ligands tested as antagonists were administered 24 min before adding the agonist. Three cycles of mixing were performed immediately after injection to ensure proper drug diffusion into the well. The effects of all compounds were expressed as the maximum change in percentage over the baseline fluorescence measured in samples treated with vehicle.

Dynamic Mass Redistribution

DMR experiments were conducted as previously described (Malfacini et al., 2018). Chinese Hamster Ovary (CHO) cells stably expressing the human kappa receptor were kindly provided by L Toll (Torrey Pines Institute for Molecular Studies, Port St. Lucie, United States). Cells were cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) supplemented with 10% (v/v) Fetal Calf Serum (FCS), 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, 15 mM HEPES. The medium was supplemented with 400 µg/ml G418 to maintain expression. Cells were seeded at a density of 45,000 cells/well in 120 µL into fibronectin-coated EnspireTM-LC 96-wells plates and cultured for 20 h to form a confluent monolayer. On the day of the experiment, cells were washed twice and maintained with assay buffer: HBSS with 20 mM HEPES, 0.01% BSA fraction V; for 90 min before the experiment. DMR was monitored in real-time with a temporal resolution of 44 s throughout the assay. Experiments were performed at 37°C, using an EnSight Multimode Plate Reader (PerkinElmer). A 5 min baseline was first established, followed by adding compounds manually in a volume of 40 µL and recording compound-triggered DMR signal for 60 min. In antagonism experiments, ligands tested as antagonists were added 30 min before agonist administration. Maximum picometers (pm) modification (peak) were used to determine agonist response after baseline normalization.

Bioluminescence Resonance Energy Transfer (BRET) Receptor-Transducer Interaction Assay

In vitro pharmacological effects of ligands at kappa opioid receptors were assessed by evaluating receptor interaction with G-protein and β-arrestin 2 with a BRET assay previously set up and validated for delta and mu (Molinari et al., 2010) and NOP (Malfacini et al., 2015) receptors.

SH-SY5Y human neuroblastoma cells permanently co-expressing the different pairs of fusion proteins were prepared using the pantropic retroviral expression system by Clontech as described previously (Molinari et al., 2010). Specifically, human kappa-RLuc fusion protein was made by linking the C-terminal of the receptor sequence without its stop codon, to the N-terminal of RLuc through a 13-mer linker peptide (RTEEQKLISEEDL) and cloned into the retroviral expression vector pQIXN (Clontech). The construction of the plasmids encoding the bovine Gβ1 and the human β-Arrestin 2 N-terminal-tagged with RGFP (Prolume, Pinetop, United States) were previously detailed (Molinari et al., 2010).

SH-SY5Y cells stably co-expressing the fusoproteins kappa-RLuc and Gβ1-RGFP or kappa-RLuc and β-arrestin 2-RGFP were grown in DMEM/F12 (1:1) medium supplemented with 10% FBS, 2 mM L-Glutamine, 100 µg/ml hygromycin B, 400 µg/ml G418, 100 units/ml penicillin G, 100 µg/ml streptomycin and 1 µg/ml Fungizone at 37°C in a humidified atmosphere with of 5% CO_2. Enriched plasma membrane samples from kappa-RLuc/Gβ1-RGFP expressing cells for receptor-G protein interaction assay were prepared by differential centrifugation and stored at −80°C until use as previously described. Total protein concentration in membrane preparations was determined by colorimetric method with Quantum Protein-BCA kit (EuroClone, Pero (MI), IT) using the multiplate reader Victor Nivo (PerkinElmer, Walthman, MA, United States). BRET assays were performed in white opaque 96 wells microplates (PerkinElmer, Walthman, MA, United States) at room temperature. In kappa opioid receptor-G protein interaction experiments, cell membranes were thawed and resuspended in PBS supplemented with 0.02% BSA, and an amount of 5 µg of total protein was dispensed in each 96 well. In kappa opioid receptor/β-arrestin 2 recruitment experiments 100,000 cells/well were seeded 24 h prior to the BRET test. On the day of the experiment, the complete medium was discarded, and cells were washed with PBS supplemented with 0.5 mM $MgCl_2$, 0.9 mM $CaCl_2$. Cells were subsequently incubated with 2 µM Prolume Purple Coelenterazine (NanoLight Technology, White Mountain, AZ; United States) for 10 min before bioluminescence reading. Agonists were added and incubated for 5 min (or 30 min where indicated) before microplate reading. In antagonism experiments, antagonists were dispensed into wells 15 min before coelenterazine incubation, Dyn A was subsequently added, and luminescence was acquired. Counts per second (CPS) were detected by Victor luminometer, emissions were selected using 405(10) and 510(30) bandpass filters for Rluc and RGFP, respectively. Acquired data were computed as BRET ratios calculated as follow:

$$\left(\frac{RGFP_{CPS}}{RLuc_{CPS}}\right)_{ligand} - \left(\frac{RGFP_{CPS}}{RLuc_{CPS}}\right)_{vehicle}$$

Agonists effects were expressed as a fraction of the maximal effect induced by Dynorphin A following blank (vehicle) subtraction.

RLuc interference experiments were performed on SH-SY5Y cell membranes prepared and quantified as described above. Because several ligands were described to directly interact with RLuc or generate unspecific luminescence artifacts (Auld et al., 2008), the amplitude of ligand-RLuc light alteration was quantified. Cells used expressed the kappa-RLuc and the β-Arrestin 2-RGFP fusoproteins. Following the membrane extraction routine, cell membranes did lose the soluble protein β-Arrestin 2-RGFP but not the kappa-RLuc allowing for precise quantification of direct RLuc light emission alteration. High concentrations of ligands were tested with membranes in the presence of CNTZ, and results were expressed in relation to vehicle/buffer set at 100% (**Supplementary Figure S1**). 15% alteration of vehicle/buffer RLuc-CNTZ emission was considered as the threshold for excluding a given compound's concentration from the concentration-response curves in following pharmacological experiments.

Data Analysis and Terminology

The pharmacological terminology is consistent with the International Union of Basic and Clinical Pharmacology (IUPHAR) recommendations (Neubig et al., 2003). Concentration-response curves to agonists were fitted to the four-parameter logistic nonlinear regression model as follows:

$$Effect = Basal + \frac{Emax - Basal}{1 + 10^{(LogEC_{50} - Log[ligand]) \times HillSlope}}$$

Curve fitting was performed using PRISM 6.0 (GraphPad Software Inc., San Diego, CA).

In antagonism experiments, concentration-response curves to Dyn A in the absence and in the presence of increasing concentrations of antagonists (Schild analysis) were performed and antagonist potency derived as pA_2 intercept to the *y*-axis of the linear regression in the Schild plot. If unsurmountable antagonist behavior was detected, the pK_B value was derived as follows: a double-reciprocal plot of equi-effective concentrations of agonist (A) in the absence (1/A) and presence (1/A') of the antagonist (B) was constructed and pK_B was derived from the equation $pK_B = \log \{(slope-1)/[B]\}$ (Kenakin, 2009).

To quantify the differences in agonist efficacies for G protein and arrestin interactions, bias factors were calculated by choosing the endogenous kappa opioid ligand Dyn A as standard unbiased ligand. For this analysis, the E_{max} and EC_{50} of the agonist were derived using a 3-parameter logistic model with slope values equal to unity. Although several agonist curves displayed slope values different from 1, refitting the curves with the parameter fixed to unity did not produce a statistically significant reduction of the goodness of fit. Under such conditions, the relative ratio $(Emax/EC_{50})_{lig}/(Emax/EC_{50})_{Dyn\ A}$ is equivalent to the relative $(\tau/K)_{lig}/(\tau/K)_{Dyn\ A}$ ratio as defined by the operational model (Black and Leff, 1983; Griffin et al., 2007). Taking ratios of these values between G protein and arrestin can cancel the common K and yield the ratio of ligand intrinsic efficacy across the two transduction proteins. Thus, the following formula was used for calculating agonist bias factors in $\log_{10}$ units:

$$\text{bias factor} = \log\left[\frac{\left(E_{max}/EC_{50}\right)_{lig}}{\left(E_{max}/EC_{50}\right)_{Dyn\ A}}\right]_{G\ prot.} - \log\left[\frac{\left(E_{max}/EC_{50}\right)_{lig}}{\left(E_{max}/EC_{50}\right)_{Dyn\ A}}\right]_{\beta-arr.}$$

Bias factors were considered significantly different from the reference ligand when a ligand's $CL_{95\%}$ did not include zero. The pharmacological terminology and computations related to biased agonism were consistent with IUPHAR recommendations (Kolb et al., 2022).

Data are expressed as mean ± sem of n experiments and were analyzed statistically using one-way analysis of variance followed by Dunnett's test for multiple comparisons. Potency values and bias factors are expressed as mean ($CL_{95\%}$). *p* values <0.05 were considered statistically significant.

RESULTS

Calcium Mobilization Assay

The endogenous peptide Dyn A, the synthetic peptide [D-Pro^{10}], and the non-peptide arylacetamide U-69,593 were chosen as standard kappa opioid agonists and tested on CHO cells stably co-expressing the kappa opioid receptor together with signaling altered chimeric G protein ($G\alpha_{qi5}$) enabling calcium mobilization assaying. In this system, all compounds evoked high maximal effects (Emax, approx. 300% fluorescence intensity units (FIU) over the baseline) and displayed high potency ranging from 9.08 for [D-Pro^{10}] to 8.09 for U-69,593 (**Figure 1Ai**). Conversely, peptide [Dmt^1,Tic^2] and non-peptide Nor-BNI antagonists did not show high stimulation of calcium flux, with [Dmt^1,Tic^2] increasing the calcium levels to 50% over the baseline only at micromolar concentrations and Nor-BNI being completely inactive (**Figure 1Aii**). Same antagonists were investigated for their capacity to block kappa opioid receptor activation in Schild protocol experiments where the effects of increasing concentrations of Dyn A were challenged with different concentrations of the two molecules. Nor-BNI produced a dextral shift in the Dyn A concentration-response curve with a progressive depression of maximal effects (**Figure 1Bi**); for this reason, the estimation of Nor-BNI potency as shown in the Schild plot (**Figure 1Bii**) was further corroborated by the approach shown in **Figure 1Biii**. Both computations returned very high potency values (pA_2 10.1 and pK_B 10.9, respectively). [Dmt^1,Tic^2] produced a rightward shift in Dyn A concentration-response curve without modifying the agonist maximal effects (**Figure 1Ci**); a pA_2 value of 6.6 was derived from these experiments (**Figure 1Cii**).

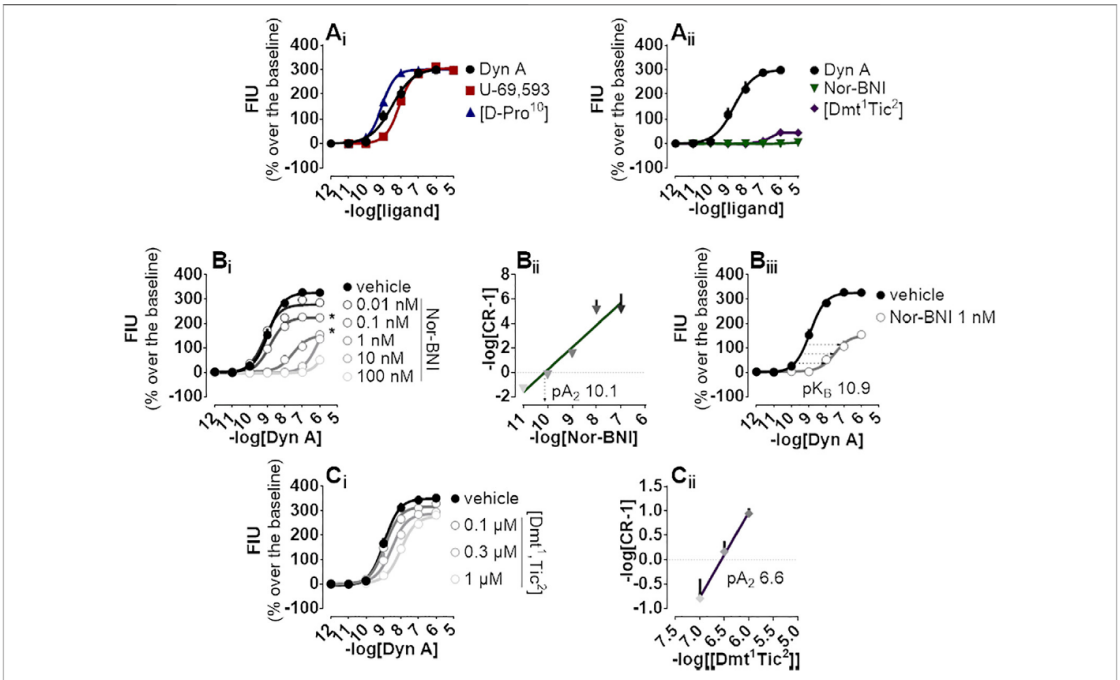

FIGURE 1 | Calcium mobilization experiments carried out in CHO cells stably expressing the human kappa opioid receptor and the $G\alpha_{qi5}$ chimeric G protein **(A_i)** Dyn A, U-69,593, and [D-Pro^{10}], and **(A_{ii})** Nor-BNI and [Dmt^1,Tic^2] concentration-response curves **(B_i)** Concentration-response curves to Dyn A in the absence and in the presence of increasing (10 p.m.—100 nM) concentrations of Nor-BNI, corresponding **(B_{ii})** Schild analysis and **(B_{iii})** computation of antagonist potency for non-competitive antagonists **(C_i)** Concentration-response curves to Dyn A in the presence of increasing concentrations (0.1—1 μM) of [Dmt^1,Tic^2] with corresponding **(C_i)** Schild analysis. Data are mean +sem of three to six independent experiments performed in duplicate and Emax were analyzed statistically using one-way analysis of variance followed by Dunnett's test for multiple comparisons. *p* values <0.05 were considered statistically significant and labelled with *.

DMR Assay

DMR is a noninvasive, unbiased, label-free approach that was employed to assess the pharmacological profile of human kappa opioid receptors expressed in CHO cells. Dyn A (**Figure 2Ai**), U-69,593 (**Figure 2Aii**), and [D-Pro^{10}] (**Figure 2Aiii**) elicited a concentration-dependent positive DMR signal, with Emax spanning from 234 to 251 pm (pm) for Dyn A and [D-Pro^{10}], respectively. Potencies for Dyn A, U-69,593, and [D-Pro^{10}] were 9.15, 8.60, and 9.84 (**Figure 2Aiv**). Nor-BNI (**Figure 2Bi**) did not modify DMR baseline while [Dmt^1,Tic^2] (**Figure 2Bii**) slightly stimulated the DMR response only at micromolar concentrations (**Figure 2Biii**).

Nor-BNI and [Dmt^1,Tic^2] antagonist properties were evaluated by Schild analysis. Comparison of representative Dyn A concentration-effect curve alone (**Figure 3Ai**) to that of Dyn A in the presence of single concentrations of Nor-BNI (1—100 nM, **Figure 3Aii-iv**) are shown. Nor-BNI concentration-dependently shifted the sigmoidal curves of Dyn A to the right without significantly affecting Emax (**Figure 3Bi**). pA_2 from Schild plot extrapolation was 10.7 (**Figure 3Bii**). Dyn A alone (**Figure 3Ci**) and following preincubation with [Dmt^1,Tic^2] at different single concentrations (0.1—1 μM, **Figure 3Cii-iv**) are shown. DMR response to Dyn A was slightly depressed by all concentrations of antagonist used (**Figure 3Di**), likely because the weak stimulatory effect of [Dmt^1,Tic^2] (as in **Figure 2Bii, Biii**) is subtracted from the baseline by the instrument software. In Schild analysis of [Dmt^1,Tic^2] against Dyn Aa pA_2 of 7.7 was calculated.

BRET Assay

A BRET approach enabling the measurement of kappa opioid receptor to G protein ($G\beta_1$) or to β-Arrestin 2 interaction was employed to test the activity of kappa opioid receptor ligands. In the kappa-G protein interaction assay, Dyn A, U-69,593, and [D-Pro^{10}] displayed similar maximal effects and potency (pEC_{50}) of 8.21, 8.52, and 8.36, respectively (**Figure 4Ai**). The interaction of the β-Arrestin 2 to the kappa opioid receptor was also promoted by Dyn A, U-69,593, and [D-Pro^{10}] with similar high maximal effects, and pEC_{50} values of 7.74, 6.72, and 8.07, respectively (**Figure 4Aii**). The computation of bias factors (towards G protein) for U-69,593 and [D-Pro^{10}] returned values of 1.22 and 0.29, respectively (**Supplementary Figure S2**).

In kappa-G protein interaction experiments, Nor-BNI (1–100 nM) shifted the concentration-effect curves to Dyn A to the right without altering maximal effects (**Figure 4Bi**). A pA_2

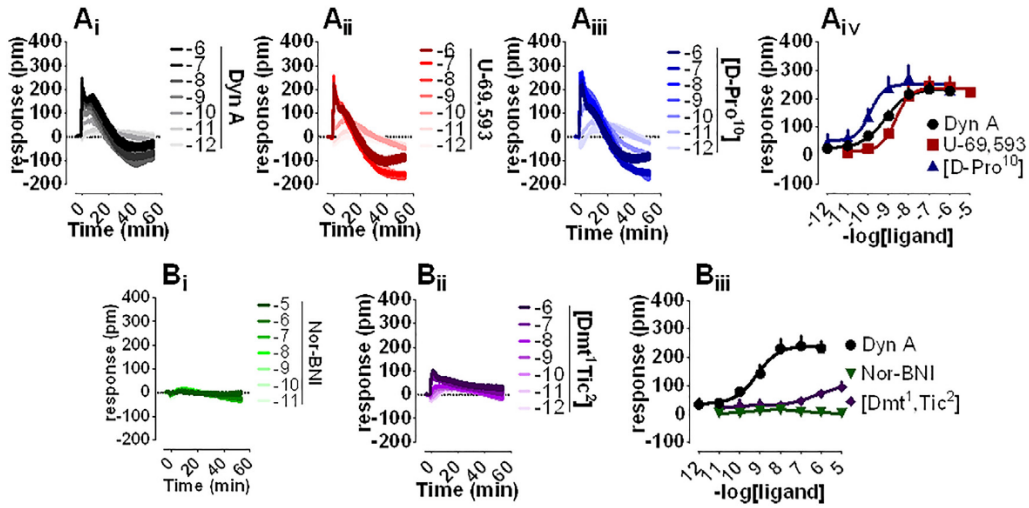

FIGURE 2 | DMR experiments in CHO cells stably expressing the human kappa opioid receptor **(A_{i-iii})** Averaged kinetics of increasing concentrations of Dyn A, U-69,593, and [D-Pro^{10}], and **(A_{iv})** corresponding concentration-response curves **(B_{i-ii})** Averaged effects of Nor-BNI and [Dmt^{1},Tic^{2}], and **(B_{iii})** corresponding concentration-response curves. Data represented are mean +sem of four to seven independent experiments performed in duplicate and Emax were analyzed statistically using one-way analysis of variance followed by Dunnett's test for multiple comparisons.

of nine was extrapolated from the Schild plot (**Figure 4Bii**). Because Nor-BNI appeared to depress the baseline of kappa-G protein interaction, and, since this assay is performed on cell membranes, GDP can be applied to investigate receptor constitutive activity (see (Vezzi et al., 2013)) for similar experiments at delta and mu opioid receptors), Nor-BNI activity was compared to that of GDP at 5 and 30 min. Experiments (**Supplementary Figure S3**, **Supplementary Table S1**) showed how Nor-BNI depression of baseline, detected also with GDP, was visible only with prolonged incubation time. Nor-BNI, in addition, generated a robust shift to the right of Dyn A response in kappa-β-Arrestin 2 interaction, with no modification of maximal effects (**Figure 4Biii**). pA_2 value obtained from the Schild plot was of 8.9 (**Figure 4Biv**). In kappa-G protein interaction experiments [Dmt^{1},Tic^{2}] concentration-dependently increased baseline (**Supplementary Figure S3**) and elicited a dextral displacement of the concentration-response curve to Dyn A without altering maximal effects (**Figure 4Ci**). Schild analysis of these results yielded a pA_2 of 7.5 (**Figure 4Cii**). In kappa-β-Arrestin 2 interaction experiments, [Dmt^{1},Tic^{2}] did not show any residual agonist activity but produced a dextral displacement of the Dyn A concentration-response curve without alteration of the agonist maximal effect (**Figure 4Ciii**); a pA_2 value of 7.3 was derived from these experiments (**Figure 4Civ**).

PWT2-Dyn A

PWT2-Dyn A is a tetrabranched derivative of Dyn A (**Figure 5Ai**) whose high-performance liquid chromatography and mass spectrometric analysis are shown in **Supplementary Figure S4**. PWT2-Dyn A was capable of evoking concentration-dependent increase of calcium mobilization in CHO cells stably expressing kappa opioid receptors and chimeric G proteins. The effects of PWT2-Dyn A were assayed up to 100 nM, and maximal effects were constrained to be the same as Dyn A (299% over the baseline) with a potency (pEC_{50}) derived for PWT2-Dyn A of 7.51 (Dyn A 8.53) (**Figure 5Bi**). CHO cells stably expressing either the mu opioid receptor and the $G\alpha_{qi5}$ chimeric G protein or the delta opioid receptor and the $G\alpha_{qG66Di5}$ chimeric G protein were employed to study the selectivity of action of PWT2-Dyn A over the mu and the delta opioid receptors. Dermorphin displayed high maximal effects (294% over the baseline) and moderate potency (pEC_{50} 7.57) in cells expressing the mu opioid receptor. Dyn A produced a stimulation (154% over the baseline) only at the highest concentration tested. PWT2-Dyn A was inactive (**Figure 5Bii**). DPDPE was tested as a standard delta opioid agonist, its maximal effects (202% over the baseline) and potency (pEC_{50} 7.74) were found high, Dyn A also displayed high maximal effects (171% over the baseline) albeit with lower (pEC_{50} 7.00) potency and PWT2-Dyn A exerted a slight (77% over the baseline) stimulatory effect only at the highest concentration tested (**Figure 5Biii**).

When Dyn A and PWT2-Dyn A activities were compared in the DMR assay with CHO cells stably expressing the kappa opioid receptor (**Figure 5Ci-iii**), PWT2-Dyn A mimicked the stimulatory effect exerted by the natural peptide Dyn A with similar maximal effects and 4-fold lower potency (pEC_{50} 8.51).

In the BRET assay, only concentrations of PWT2-Dyn A up to 10 nM could be tested because of unclear alteration of the RLuc emitted light (**Supplementary Figure S1**). Nevertheless, 10 nM PWT2-Dyn A reached the maximal effects of Dyn A in the kappa-G protein (**Figure 5Di**) and kappa-β-Arrestin 2 (**Figure 5Dii**) interaction assays, with derived potency values 3-fold lower than Dyn A in both assays.

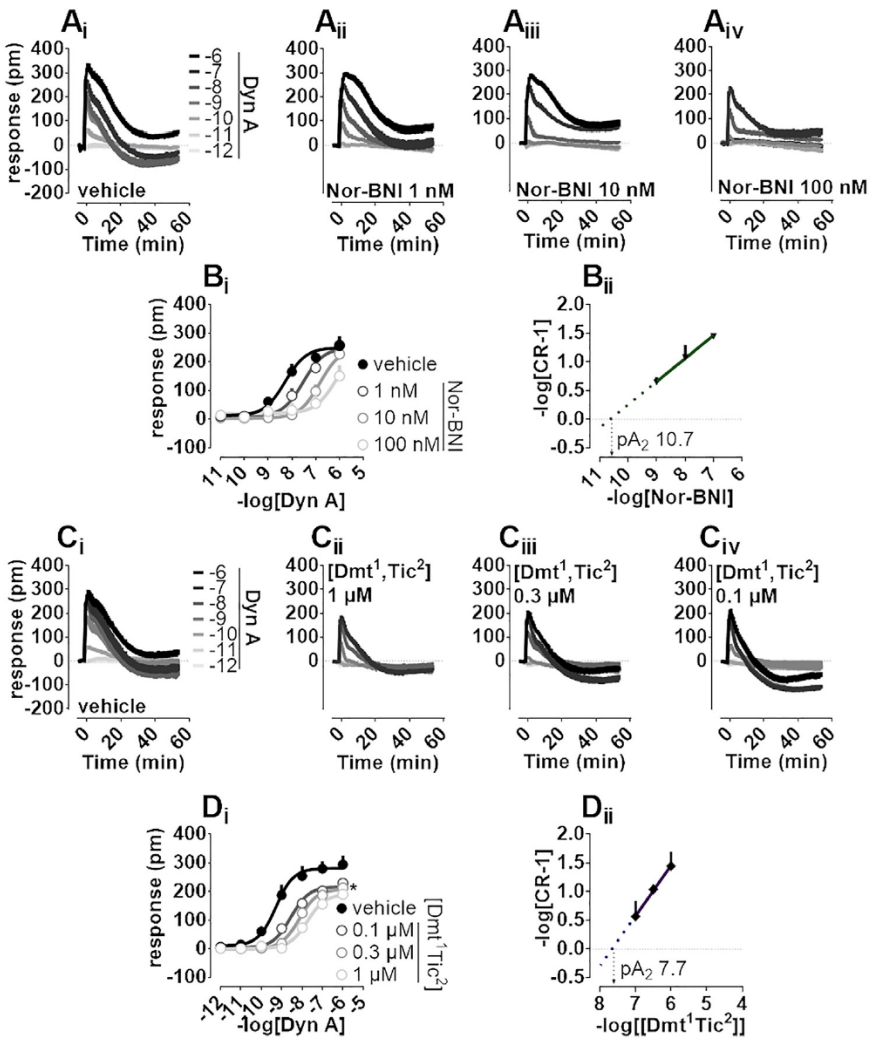

FIGURE 3 | DMR experiments in CHO cells stably expressing the human kappa opioid receptor **(A_{i-iv})** Averaged kinetic traces of increasing (1 p.m.—1 μM) concentrations of Dyn A in the absence and in the presence of different (1–100 nM) concentrations of Nor-BNI, corresponding **(B_i)** concentration-response curves and **(B_{ii})** Schild analysis **(C_{i-iv})** Averaged kinetic traces of increasing Dyn A concentrations in the absence and in the presence of different (0.1–1 μM) concentrations of [Dmt^1,Tic^2] with corresponding **(D_i)** concentration-response curves and **(D_{ii})** Schild analysis. Data represented are mean +sem of five to seven independent experiments performed in duplicate and Emax were analyzed statistically using one-way analysis of variance followed by Dunnett's test for multiple comparisons. *p* values <0.05 were considered statistically significant and labelled with *.

Dyn A-Palmitic

A palmitoylated derivative of Dyn A, Dyn A-palmitic (**Figure 6Ai**), was examined. Dyn A-palmitic high-performance liquid chromatography and mass spectrometric analysis are shown in **Supplementary Figure S5**. In calcium mobilization studies performed with kappa receptor-expressing cells, Dyn A-palmitic displayed high maximal effects similar to that of Dyn A with only 2-fold loss of potency (**Figure 6Bi**). The selectivity of Dyn A-palmitic over the mu and the delta opioid receptors was assayed as for PWT2-Dyn A. Dyn A-palmitic, mimicked the stimulatory effects of dermorphin, with an approximately 6-fold lower potency (**Figure 6Bii**). At delta opioid receptor-expressing cells Dyn A-palmitic was 10-fold less potent than DPDPE (**Figure 6Biii**).

Dyn A-palmitic was tested on the DMR assay together with Dyn A (**Figure 6Ci, Cii**), both compounds elicited high maximal effects in CHO cells expressing the kappa opioid receptor, with Dyn A-palmitic showing 6-fold lower potency than Dyn A (**Figure 6Ciii**).

In the BRET assay for kappa-G protein interaction, Dyn A and Dyn A-palmitic concentration-response curves were superimposable (potency ~6 nM) (**Figure 6Di**). In kappa-β

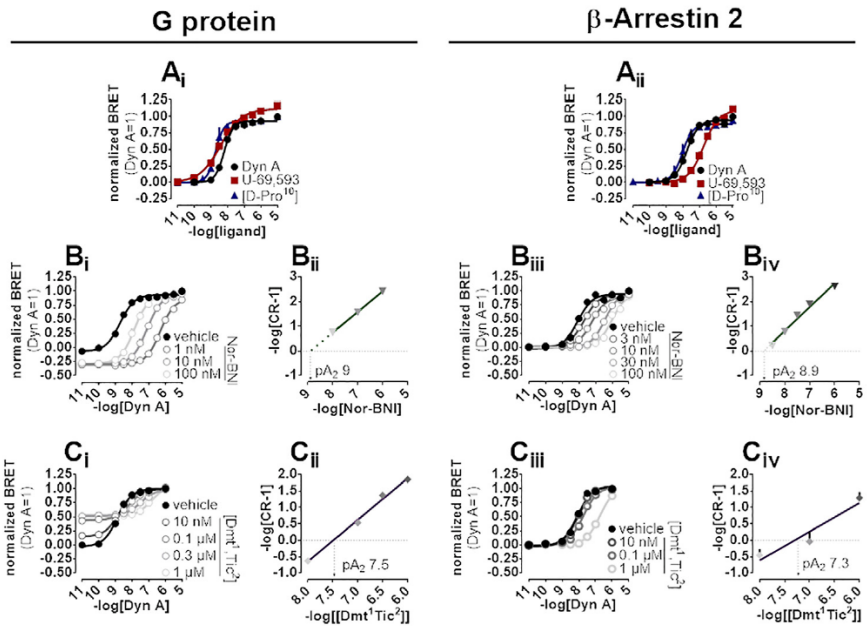

FIGURE 4 | BRET experiments in SH-SY5Y cells stably expressing the human kappa opioid-RLuc tagged receptor together with either the $G\beta_1$-RGFP or the β-Arrestin 2-RGFP fusoprotein. Concentration-response curves to Dyn A, U-69,593, and [D-Pro^{10}] on the stimulation of kappa-G protein **(A_i)** and kappa-β-Arrestin 2 **(A_{ii})** interaction. Concentration-response curves to Dyn A in the absence and in the presence of increasing concentrations of Nor-BNI on the stimulation of kappa-G protein **(B_i)** and kappa-β-Arrestin 2 **(B_{iii})** interactions with corresponding Schild plots **(B_{ii}** and **B_{iv}**, respectively**)**. Concentration-response curves to Dyn A in the absence and in the presence of increasing concentrations of [Dmt^1,Tic^2] on the stimulation of kappa-G protein **(C_i)** and kappa-β-Arrestin 2 **(C_{iii})** interaction, with corresponding Schild analysis **(C_{ii}** and **C_{iv}**, respectively**)**. Data represented are mean +sem of three to five independent experiments performed in duplicate.

Arrestin-2 interaction experiments and despite mimicking the stimulatory effects of Dyn A with similar maximal effects, Dyn A-palmitic showed 7-fold lower potency than the natural peptide (**Figure 6Dii**). A bias factor (towards G protein) of 0.74 was derived from these experiments (**Supplementary Figure S2**).

All pharmacological parameters with corresponding $CL_{95\%}$ or sem are summarized in **Table 1**. Results obtained in terms of biased agonism are plotted as bias plot and bias factors bar diagrams in **Supplementary Figure S2**.

DISCUSSION

The kappa opioid receptor controls important biological functions, and its pharmacological modulation has therapeutic potential. For instance, selective kappa receptor antagonists promote robust anxiolytic and antidepressant-like effects and can also be investigated as innovative drugs to treat substance use disorders (Lutz and Kieffer, 2013; Carlezon and Krystal, 2016). Whilst, kappa receptor agonists display analgesic/antinociceptive and antipruritic (e.g. the Japanese marketed nalfurafine (Inui, 2015)) properties, clinical development is limited by important side effects such as dysphoria, sedation, hallucinations, and diuresis. To increase the therapeutic index of kappa receptor agonists, different strategies have been proposed, including G protein-biased agonists, peripherally restricted molecules, and mixed opioid receptor agonists (Paton et al., 2020).

The aim of this study was twofold, establishing a panel of assays to finely study kappa opioid receptor pharmacology and characterize novel ligands: PWT2-Dyn A and Dyn A-palmitic. We, therefore, deployed an array of assays to investigate the pharmacological profile of the human kappa opioid receptor in recombinant cells. The kappa opioid receptor natively couples to Gi/o heterotrimeric G proteins (Paton et al., 2020); therefore, we could measure calcium mobilization (a prototypical Gq signal) only adopting chimeric Gq/i proteins as described before by us (Camarda and Calo, 2013) and others (Zhang et al., 2012). DMR, an integrated, unbiased approach to assess real-time activation of intracellular signaling, was also employed as previously described for different GPCRs (Schroder et al., 2011), including opioid receptors (Morse et al., 2011; Grundmann et al., 2018). Finally, as previously reported for other opioid receptors (Molinari et al., 2010; Vezzi et al., 2013; Malfacini et al., 2015), two BRET approaches for measuring kappa-G protein and kappa-β-Arrestin 2 interaction were used to further investigate the pharmacology of kappa ligands, and eventually estimate their bias factor. Collectively, the pharmacological fingerprint of the kappa opioid receptor, obtained with potent and selective agonists and antagonists, is in line to that reported in the literature (Alexander et al., 2021). Novel ligands, PWT2-Dyn A and Dyn A-palmitic, showed similar pharmacology to that of

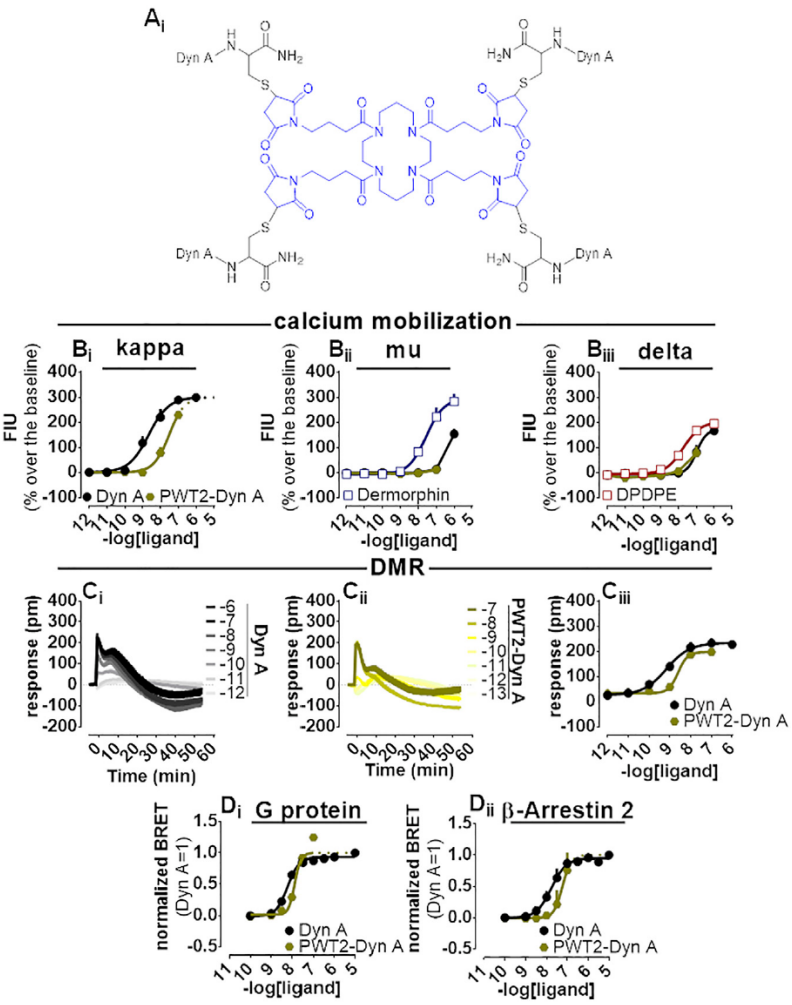

FIGURE 5 | Pharmacological characterization of PWT2-Dyn A. Molecular structure **(A_i)**. Calcium mobilization experiments in CHO stably expressing the human kappa **(B)**, mu **(Bii)i** and delta **(Bii)** opioid receptor and chimeric G proteins, concentration-response curves to Dyn A and PWT2-Dyn A. Averaged DMR traces obtained in CHO cells stably expressing the human kappa opioid for increasing concentration of Dyn A **(C_i)** and PWT2-Dyn A **(C_{ii})**, with corresponding concentration-response curves **(C_{iii})**. Concentration response curves to Dyn A and PWT2-Dyn A performed with a BRET assay on SH-SY5Y cells stably expressing the human kappa opioid-RLuc tagged receptor together with either the Gβ_1-RGFP **(D_i)** or the β-Arrestin 2-RGFP fusoprotein **(D_{ii})**. Data are mean +sem of three to six independent experiments performed in duplicate.

the parental natural peptide Dyn A although with slight decrease of potencies. In addition, Dyn A-palmitic showed a significant bias towards G protein.

Agonists Pharmacological Fingerprint

Collectively, in our assays, Dyn A, U-69,593, and [D-Pro^{10}] behaved as full agonists of the human kappa opioid receptor with the following rank order of potency [D-Pro^{10}] > Dyn A ≥ U-69,593, which is in line with several studies employing different assays and preparations (cells and isolated tissues), and multiple isoforms of the kappa opioid receptor (human, mouse, and guinea pig) (Gairin et al., 1984; Lahti et al., 1985; Gairin et al., 1988b; Meng et al., 1993; Yasuda et al., 1993; Soderstrom et al., 1997). Dyn A elicited robust activation of the kappa opioid receptor with potency ranging from 1 to 6 nM, except for the kappa-β-arrestin 2 interaction assay with a slightly lower potency of 18 nM. Similar results were obtained in calcium mobilization studies (Camarda and Calo, 2013). For stimulation of [^{35}S]GTPγS binding, potency values obtained were approximately one order of magnitude higher (Zhu et al., 1997). U-69,593 potencies ranked from 3 to 8 nM with 191 nM in the kappa-β-arrestin 2 interaction assay, demonstrating a robust bias towards G protein activation. Literature data showed similar potency values in receptor

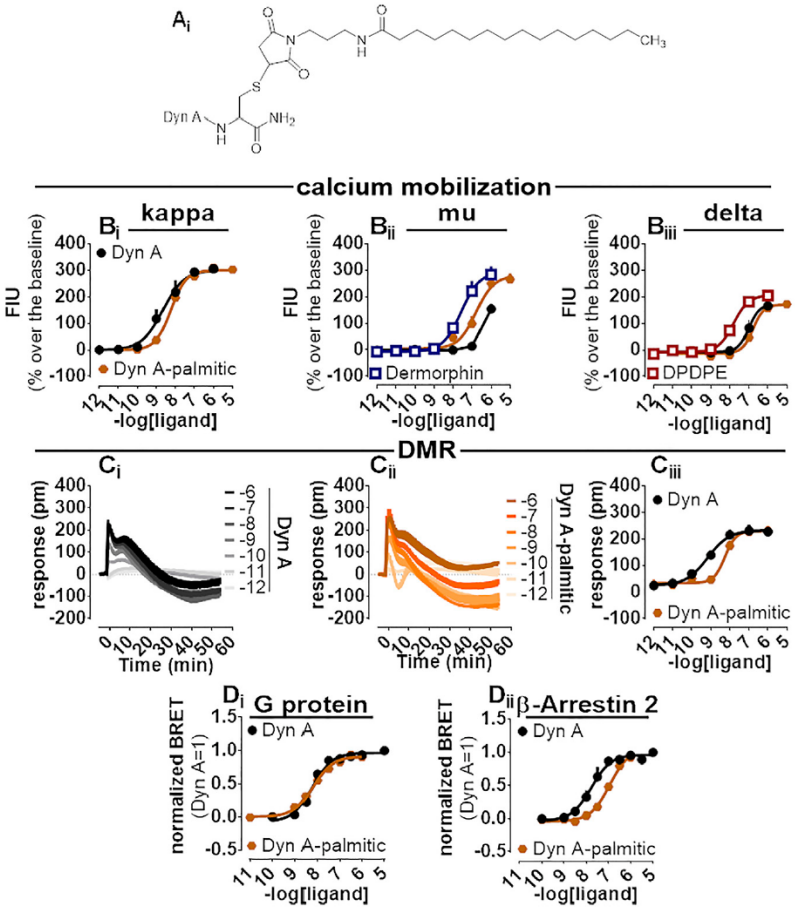

FIGURE 6 | Pharmacological characterization of Dyn A-palmitic. Molecular structure **(A_i)**. Calcium mobilization experiments in CHO cells stably expressing the human kappa **(B_i)**, mu **(B_{ii})**, and delta **(B_{iii})** opioid receptors and chimeric G proteins. Concentration-response curves to Dyn A and Dyn A-palmitic. Averaged DMR traces obtained in CHO cells stably expressing the human kappa opioid for increasing concentration of Dyn A **(C_i)** and Dyn A-palmitic **(C_{ii})**, with corresponding concentration-response curves **(C_{iii})**. Concentration response curves to Dyn A and Dyn A-palmitic performed with a BRET assay on SH-SY5Y cells stably expressing the human kappa opioid-RLuc tagged receptor together with either the $G\beta_1$-RGFP **(D_i)** or the β-Arrestin 2-RGFP fusoprotein **(D_{ii})**. Data are mean +sem of three to six independent experiments performed in duplicate.

binding assay of 4 nM, and forskolin stimulated inhibition of cAMP of 17 nM (Katsumata et al., 1996). However, G protein-biased behavior of U-69,593 is not corroborated by literature findings. In CHO cells expressing the human kappa receptor, U-69,593 displayed similar potency and efficacy in [^{35}S]GTPγS stimulation and β-arrestin 2 recruitment experiments; however, the effects of U-69,593 were not compared to those of Dyn A (Ho et al., 2018). In the study by White and co-workers (White et al., 2014), U-69,593 has been classified as an unbiased kappa agonist, but Salvinorin A rather than Dyn A was used as a reference ligand. Finally, U-69,593 was also reported to be biased for internalization versus [^{35}S]GTPγS stimulation (DiMattio et al., 2015). These inconsistencies underline the need of adopting more stringent rules in the design and reporting of biased agonism studies; these rules have been recently made available by IUPHAR (Kolb et al., 2022).

[D-Pro^{10}] is a relatively poorly studied, highly potent Dyn A derivative. Developed in 1984 (Arimitsu et al., 1977), non-amidated [D-Pro^{10}]Dyn(1-11) displayed a 7-fold higher affinity to the kappa opioid receptor (detected with [^{3}H] Bremazocine binding) than Dyn A and showed 62- and 233-fold selectivity over the mu and delta opioid receptors, respectively. This peptide has been used as a probe to visualize kappa-opioid receptor sites in autoradiographic studies (Jomary et al., 1988). Intracerebroventricular administration of [D-Pro^{10}] Dyn(1-11) in mice did not show any activity against thermal stimulus (tail-flick), but produced a dose-dependent antinociceptive effect in the acetic acid-induced writhing model of visceral pain, in line with a kappa-driven agonist activity (Gairin et al., 1988a). Our results confirm the high potency and full agonist activity of this peptide as a kappa agonist. In addition, the present findings suggest that

TABLE 1 | Pharmacological parameters and relative $CL_{95\%}$ for Dyn A, U-69,593, [D-Pro^{10}], Nor-BNI, [Dmt^{1}, Tic^{2}], PWT2-DynA, and DynA-Palmitic in the indicated assays.

	Calcium Mobilization			DMR			BRET					
							G Protein			β-Arrestin 2		
	pEC_{50} ($CL_{95\%}$)	α	pA_2 ($CL_{95\%}$)	pEC_{50} ($CL_{95\%}$)	α	pA_2 ($CL_{95\%}$)	pEC_{50} ($CL_{95\%}$)	A	pA_2 ($CL_{95\%}$)	pEC_{50} ($CL_{95\%}$)	α	pA_2 ($CL_{95\%}$)	Bias factor[a] ($CL_{95\%}$)
Dyn A	8.53 (7.94–9.12)	1.00	–	9.15 (8.56–9.74)	1.00	–	8.21 (8.12–8.30)	1.00	–	7.74 (7.33–8.15)	1.00	–	0.00
U-69,593	8.09 (7.93–8.26)	1.00	–	8.60 (8.21–8.98)	1.01	–	8.52 (8.27–8.78)	1.17	–	6.72 (6.37–7.06)	1.12	–	1.22 (0.87–1.54)
[D-Pro^{10}]	9.08 (8.93–9.23)	1.00	–	9.84 (9.31–10.37)	1.07	–	8.36 (6.54–10.19)	1.00	–	8.07 (7.66–8.47)	0.95	–	0.29 (-0.06–0.64)
Nor-BNI	inactive		10.1[b] (9.41-11.51)	inactive		10.7 (9.42–11.98)	8.40 (6.46–10.35)	- 0.11	9 (8.65–9.20)	inactive		8.9 (8.50–9.38)	–
[Dmt^{1},Tic^{2}]	-6.9	0.15	6.6 (6.16–7.14)	7.11 (5.61–8.61)	0.47	7.7 (7.11–8.29)	6.95 (6.14–7.75)	0.35	7.5 (5.35–9.65)	inactive		7.3 (6.5–8.1)	–
PWT2-DynA	7.51 (6.91–8.11)	~1.00	–	8.51 (9.3–10.37)	0.84	–	7.73 (7.41–8.04)	~1.00	–	6.66 (6.30–7.02)	~1.00	–	~0.18[c]
DynA-Palmitic	8.24 (7.91–8.56)	1.01	–	8.38 (8.06–8.70)	0.99	–	8.17 (7.94–8.40)	0.90	–	6.90 (6.76–7.04)	0.92	–	0.74 (0.52–0.96)

α is the intrinsic activity of a ligand: calculated as the maximal effect of a given agonist divided that of Dyn A.

[a]*Bias factors were calculated using the naturally occurring peptide Dyn A as unbiased agonist.*

[b]*Nor-BNI, antagonist potency in the calcium mobilization assay was also determined with the double-reciprocal approach as described in the method section and gave a pK_B of 10.9 (10.55-11.25).*

[c]*Because of interaction of PWT2-Dyn A with RLuc, light, in BRET, experiments Emax was set equal to one and an approximation of bias factor computed (without error estimation).*

the [D-Pro^{10}] chemical modification does not affect the unbiased agonist features of the naturally occurring peptide Dyn A.

Antagonists Pharmacological Fingerprint

As far as antagonism is concerned, Dyn A concentration-response curve was challenged by increasing concentrations of Nor-BNI and [Dmt^{1},Tic^{2}] in all assays adopted. Nor-BNI displayed potency values ranging 0.01–1.26 nM. [Dmt^{1},Tic^{2}] potency, was lower and ranged from 20 to 251 nM. The pharmacological activity of Nor-BNI is in line with the literature, its affinity reported is approximately 1 nM (Simonin et al., 1995) and potency 0.1 nM (Zhu et al., 1997) (Birch et al., 1987). Regarding the type of antagonism, because the calcium mobilization assay was the only one showing an unsurmountable type of antagonism for Nor-BNI, we still classify Nor-BNI as a competitive antagonist. Unsurmountable/noncompetitive-like behaviors can be measured when antagonist-receptor complexes are reversible per se but dissociate too slowly that only a fraction of the receptors are accessible to subsequent stimulation by the agonist. In the calcium mobilization assay, the short time in which agonist effects are measured does not allow equilibration to be reached, especially when the antagonist slowly dissociates from the receptor (Charlton and Vauquelin, 2010). In fact, Nor-BNI is a very long-lasting antagonist *in vivo*, displaying an irreversible-like behavior (Jones and Holtzman, 1992) [Dmt^{1},Tic^{2}] was developed in 1998 (Guerrini et al., 1998) and showed affinity values of 3.3—4.3 nM, with equal affinity at the mu opioid receptor and 10-fold higher affinity at the delta opioid receptor. Bioassay experiments on isolated tissues gave antagonist potency values of 90 and 8 nM for this peptide in guinea pig ileum and rabbit jejunum, respectively. The present results demonstrated that [Dmt^{1},Tic^{2}] is actually a low efficacy (α 0.15–0.3) partial agonist at the human kappa receptor; of note the peptide behaved as a pure antagonist only in the kappa-β-arrestin 2 interaction assay. As expected partial agonist potency of [Dmt^{1},Tic^{2}] was close to antagonistic potency values measured in the same assay. Compounds like [Dmt^{1},Tic^{2}] characterized by mixed opioid receptor activity and different degrees of efficacy are currently considered of great interest (Paton et al., 2020); clearly further studies are needed to investigate the beneficial versus unwanted effects elicited *in vivo* by this opioid receptor ligand.

Inverse Agonism

In kappa-G protein interaction experiments, Nor-BNI not only antagonized the effects of Dyn A but produced a concentration-dependent reduction in the baseline, thus behaving as an inverse agonist. Interestingly, in line with the slow kinetics (see before) of this molecule, this effect was evident only with prolonged incubation time. In addition, kappa opioid receptor constitutive activity is confirmed by experiments with GDP that, being capable of inhibiting the activated G protein, can elicit a condition of virtual zero receptor activation (Vezzi et al., 2013). Since the maximal inhibitory effect of Nor-BNI is similar to that of GDP, Nor-BNI should be classified as a full inverse agonist. As expected, the pEC_{50} of Nor-BNI studied as inverse agonist was close to the pA_2 obtained investigating the molecule as antagonist. The present results corroborate previous findings obtained in [^{35}S]GTPγS binding studies performed with HEK cells expressing the kappa receptor both in term of inverse agonist behavior of Nor-BNI and amount of kappa receptor constitutive activity (10–15% of basal values) (Wang et al., 2007). The liability of the kappa receptor to display constitutive activity seems to be similar to that of the mu receptor and much lower to that of the delta opioid receptor (Wang et al., 2007; Vezzi et al., 2013). Indeed, constitutive activity at GPCRs was first described in cells expressing delta receptors (Costa and Herz, 1989). As far as the possible physiological role of kappa receptor constitutive activity is concerned, little information is available in the literature; however, there is evidence suggesting that constitutively active kappa receptors are expressed in the ventral tegmental area (Polter et al., 2017) and in the medial prefrontal cortex (Sirohi and Walker, 2015) where may regulate response to stress in relation to substance use disorders and impulsivity, respectively.

PWT2-Dyn A and Dyn A-Palmitic

Finally, we assessed how PWT2-Dyn A and Dyn A-palmitic behave in our pharmacological assays. PWT stands for peptide welding technology, and it is a relatively novel approach to generate with high yield and purity tetrabranched peptide molecules (Guerrini et al., 2014). PWT derivatives of nociceptin/orphanin FQ and related peptides, opioid peptides, tachykinins, and neuropeptide S were generated and characterized pharmacologically in previous studies (reviewed in (Calo et al., 2018)). *In vitro* these PWT derivatives mimicked the effects of the native peptides with no major changes of affinity, pharmacological activity, potency, or selectivity of action. However, *in vivo* studies demonstrated that PWT peptides are generally characterized by increased potency and, more importantly, long-lasting effects (Calo et al., 2018). Accordingly, in this study, PWT2-Dyn A showed pharmacology very similar to Dyn A, and similar selectivity for kappa over mu and delta opioid receptors. In addition, although caution should be exercised in evaluating PWT2-Dyn A data in the BRET assay (i.e. alteration of RLuc light), PWT2-Dyn A seemed to maintain the unbiased features of Dyn A. Clearly, further studies are needed to evaluate the *in vivo* actions of PWT2-Dyn A, assessing the usefulness of this kappa receptor ligand as a novel research tool.

We synthesized and characterized the palmitoylated derivative of Dyn A (Dyn A-palmitic). In the frame of a previous structure-activity relationship study on nociceptin/orphanin FQ, C-terminus palmitoylation has been identified as the chemical modification leading to the highest increase in G protein bias at the NOP receptor (Pacifico et al., 2020). In the same study, C-terminal palmitoylation was also applied at other opioid peptides including dermorphin, deltorphin A, and Leu-enkephalin, which were tested at mu and delta receptors. Palmitoylated peptides consistently displayed high potency in receptor-G protein interaction experiments while their effects in receptor-β-arrestin 2 interaction experiments were variable: they behaved as G protein biased agonists at NOP and delta receptors and as unbiased agonists at the mu receptor (Pacifico et al., 2020). Results obtained at the kappa opioid receptor with Dyn A-palmitic are therefore only partially in line with the findings

mentioned above. In fact, C-terminal palmitoylation of Dyn A generated a kappa selective full agonist, but its potency was slightly lower than that of the native peptide. On the other hand, Dyn A-palmitic displayed a statistically significant bias factor towards G protein at kappa receptors, similar to that reported for palmitoylated peptides at NOP and delta (but not mu) receptors. Thus, the present results corroborated previous findings suggesting that C-terminal palmitoylation is a valuable strategy for altering the signaling properties of opioid receptor ligands, although this is not always associated with increased potency. The mechanism by which C-terminal modifications of peptide agonists for opioid receptors promote biased agonism toward G protein is at present unknown. The discussion of this specific issue and some speculative considerations have been reported in (Pacifico et al., 2020). Dyn A-palmitic could be added to the available research tools for investigating functional selectivity and its potential in drug discovery in the opioid receptor field (Dogra and Yadav, 2015; Gomes et al., 2020; Che et al., 2021; French and van Rijn, 2022).

CONCLUSION

The overall results obtained in the different assays with standard kappa receptor ligands are in line with literature findings both in terms of rank order of potency of agonists and potency of selective and competitive antagonists. In addition, the use of multiple assays and standard ligands allows detailed pharmacological investigation of activity and classification of ligands as full and partial agonists, neutral antagonists, and inverse agonists. We generated and studied PWT2-Dyn A and Dyn A-palmitic, which demonstrated selective kappa receptor full agonist behavior. Moreover, BRET receptor-transducer interaction studies suggested that PWT2-Dyn A acts as an unbiased agonist while Dyn A-palmitic as an agonist biased toward G protein. Further studies should assess the features of these novel compounds *in vivo* in animal models of pain and pruritus, conditions in which the selective activation of kappa receptors elicits beneficial effects.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

Conceptualization: GC', PM Formal analysis: CS, DM, CR, RG Funding acquisition: CR, RG, GC', PM Investigation: CS, DM, MA, FD, EM, VA, CR, PM Resources: EM, VA, RG, PM Supervision: CR, GC', PM Writing—original draft: DM Writing—review and editing: DM, CR, RG, GC', PM.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2022.873082/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

SUPPORTING INFORMATION

Pharmacology of kappa opioid receptors: novel assays and ligands

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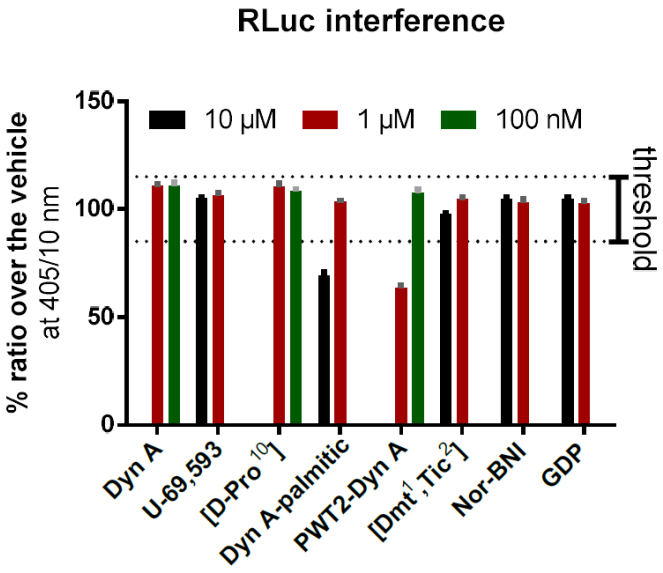
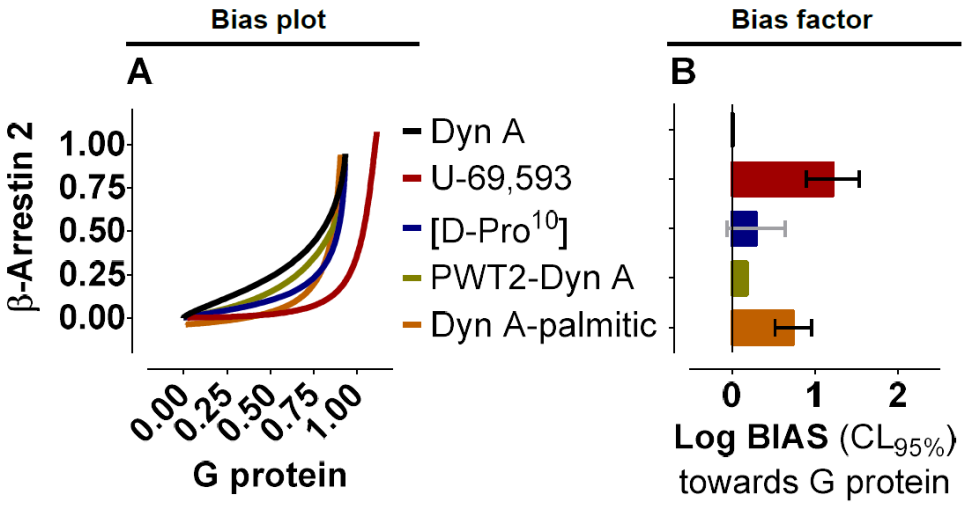


Supplementary Figure 1. Compound interference with the RLuc light. Membranes taken from SH-SY5Y cells stably expressing the human kappa opioid-RLuc tagged receptor together with the β-Arrestin 2-RGFP fusoprotein.
Data are mean + sem of 3 experiments performed in duplicate.



Supplementary Figure 2. Bias plot and Bias factors from G protein and β-Arrestin 2 BRET data.

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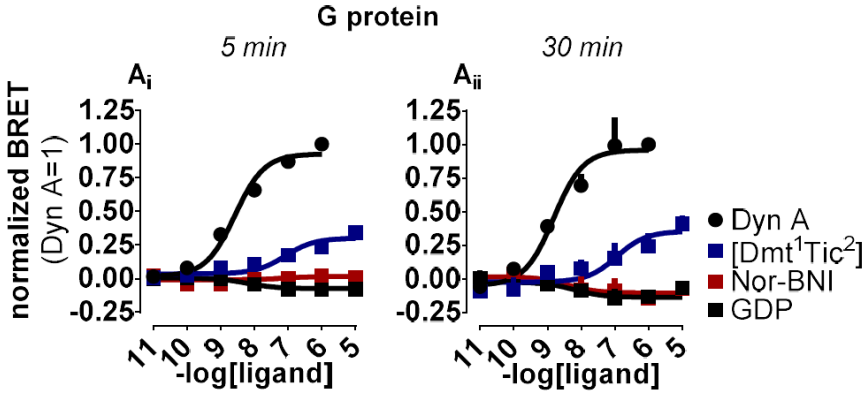
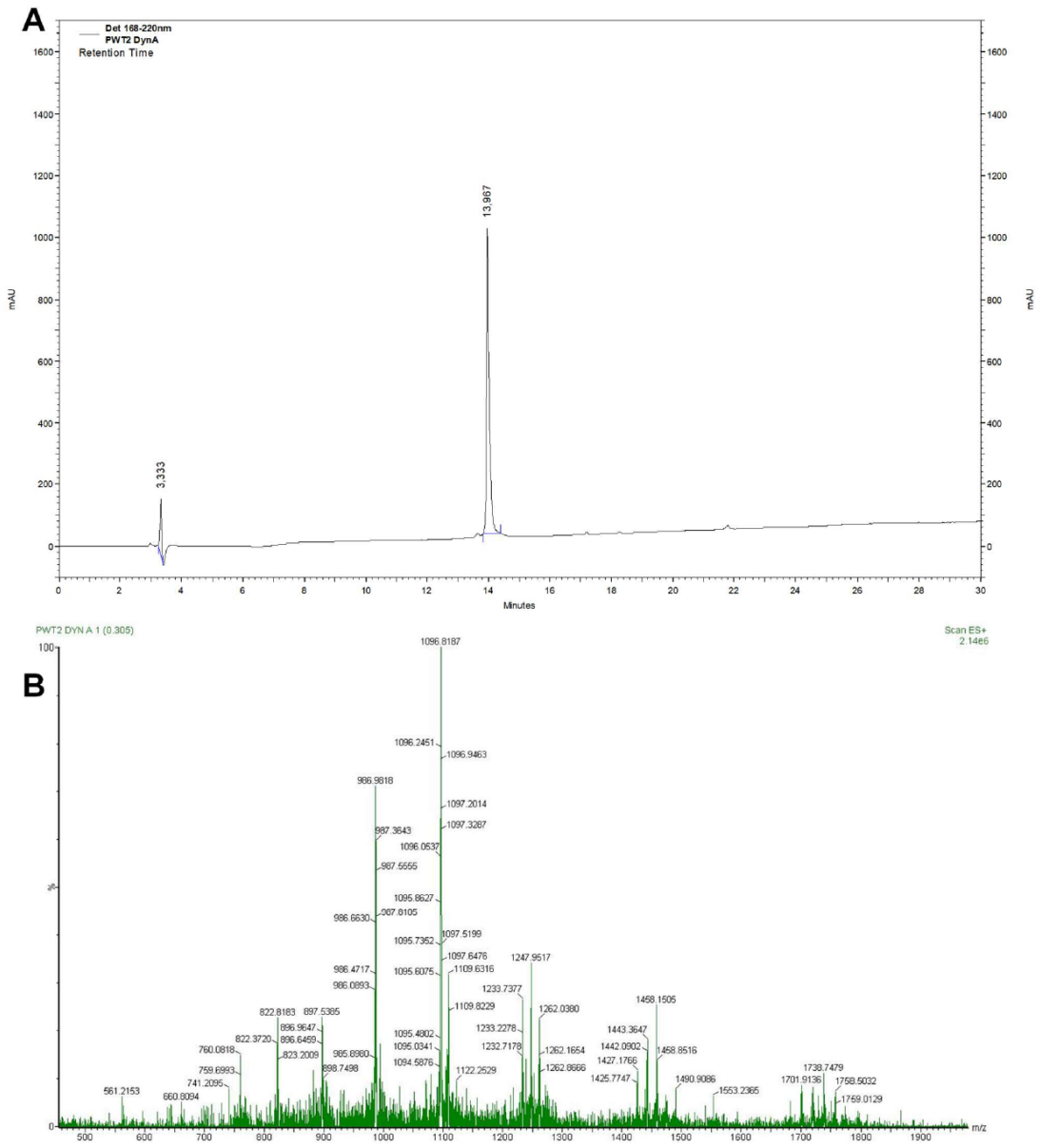


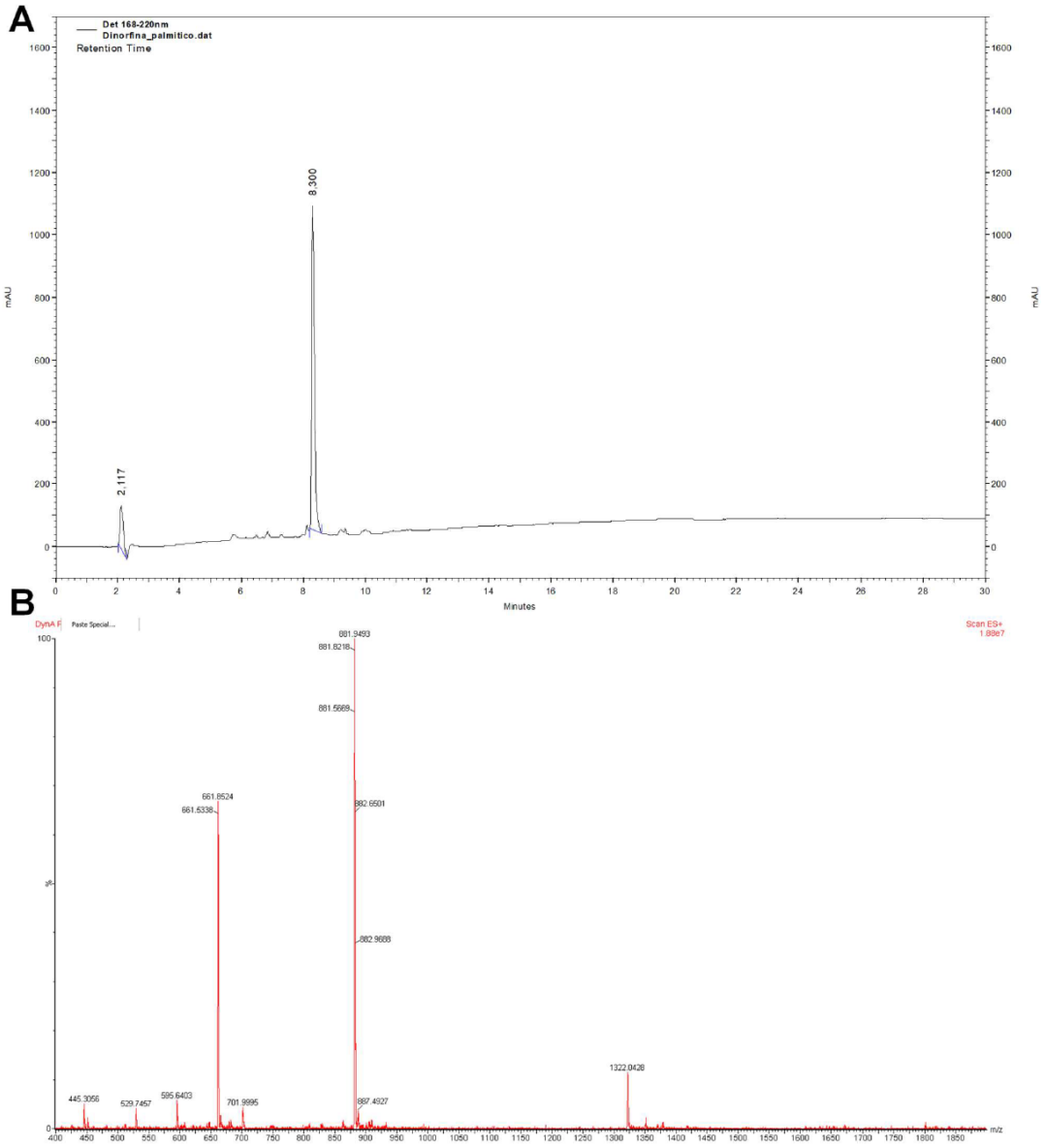
Supplementary Figure 3. BRET experiments on membrane taken from SH-SY5Y cells stably expressing the human kappa opioid-RLuc tagged receptor together with the Gβ$_1$-RGFP fusoprotein. Concentration-response curves to Dyn A, [Dmt^1,Tic^2], Nor-BNI, and GDP. BRET measurements were made out 5 min (**Ai**) and 30 min (**Aii**) after compound addition. Data shown are mean + sem of at least 3 independent experiments performed in triplicate.

Supplementary Table 1. Pharmacological parameters obtained at 5 min and 30 min in BRET experiments on membrane taken from SH-SY5Y stably expressing the human kappa opioid-RLuc tagged receptor together with the Gβ$_1$-RGFP fusoprotein.

	5 min		30 min	
	pEC$_{50}$ (CL$_{95\%}$)	**Emax ± sem**	**pEC$_{50}$ (CL$_{95\%}$)**	**Emax ± sem**
Dyn A	8.58 (8.43 - 8.72)	1.00	8.81 (8.45 - 9.17)	1.00
[Dmt^1,Tic^2]	7.09 (6.68 - 7.49)	0.30 ± 0.02	6.95 (6.14 - 7.75)	0.35 ± 0.06
Nor-BNI	inactive		8.40 (6.46 - 10.35)	- 0.11 ± 0.04
GDP	8.20 (7.29 - 9.11)	- 0.07 ± 0.01	8.21 (6.41 - 10.01)	- 0.14 ± 0.03



Supplementrary Figure 4. Analytical HPLC chromatogram (**A**) and Electrospray ionization (ESI) mass spectra of PWT2-Dyn A; MW calculated Da: 9859.48 (**B**).



Supplementary Figure 5. Analytical HPLC chromatogram (**A**) and electrospray ionization (ESI) mass spectra of Dyn A-palmitic; MW calculated Da: 2642.26 (**B**).

2.2 Novel mixed NOP/opioid peptide agonists

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Novel Mixed NOP/Opioid Receptor Peptide Agonists

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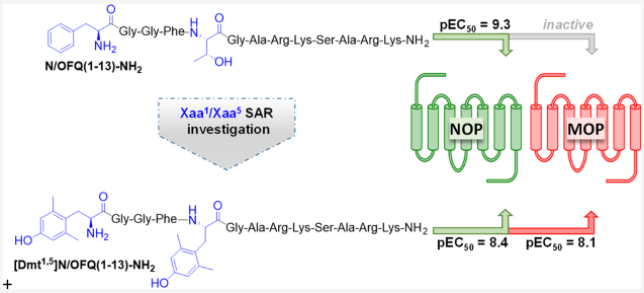

ABSTRACT: The nociceptin/orphanin FQ (N/OFQ)/N/OFQ receptor (NOP) system controls different biological functions including pain and cough reflex. Mixed NOP/opioid receptor agonists elicit similar effects to strong opioids but with reduced side effects. In this work, 31 peptides with the general sequence [Tyr/Dmt[1],Xaa[5]]N/OFQ(1-13)-NH_2 were synthesized and pharmacologically characterized for their action at human recombinant NOP/opioid receptors. The best results in terms of NOP versus mu opioid receptor potency were obtained by substituting both Tyr[1] and Thr[5] at the N-terminal portion of N/OFQ(1-13)-NH_2 with the noncanonical amino acid Dmt. [Dmt[1,5]]N/OFQ(1-13)-NH_2 has been identified as the most potent dual NOP/mu receptor peptide agonist so far described. Experimental data have been complemented by in silico studies to shed light on the molecular mechanisms by which the peptide binds the active form of the mu receptor. Finally, the compound exerted antitussive effects in an in vivo model of cough.

INTRODUCTION

Nociceptin/orphanin FQ (N/OFQ; FGGFTGARKSARKLANQ) is the endogenous ligand of the N/OFQ peptide (NOP) receptor.[1,2] N/OFQ and the NOP receptor display high structural homology with peptides and receptors of the opioid family but distinct pharmacology.[3] The N/OFQ-NOP receptor system controls several biological functions at both central and peripheral levels including pain transmission, mood and anxiety, food intake, learning and memory, locomotion, cough and micturition reflexes, cardiovascular homeostasis, intestinal motility, and immune responses.[4]

The effects of N/OFQ and selective NOP agonists in analgesiometric assays are complex depending on the dose, administration route, type of pain, and animal species.[5,6] On the contrary, strong and consistent experimental evidence suggests that the simultaneous activation of NOP and opioid receptors elicits synergistic analgesic effects.[6,7] On these bases, mixed NOP/opioid receptor agonists (cebranopadol,[8,9] AT-121,[10] BU10038,[11] and BPR1M97[12]) have been developed and investigated for their antinociceptive properties. It was consistently demonstrated that these drugs elicit similar analgesic effects to strong opioids but with substantially reduced side effects including respiratory depression, tolerance, and abuse liability (see the recent review by Kiguchi et al.[13]).

Other ligands targeting multiple opioid receptors have been studied.[14] For example, dual-acting mu agonist/delta antagonist peptidomimetics demonstrated to produce antinociception in vivo with reduced tolerance liability compared with morphine.[15,16] Moreover, mixed kappa agonist/mu partial agonist ligands have been investigated as potential treatment agents for cocaine and other psychostimulant abuses.[17] Finally, mixed kappa agonist/delta antagonist ligands have been developed as

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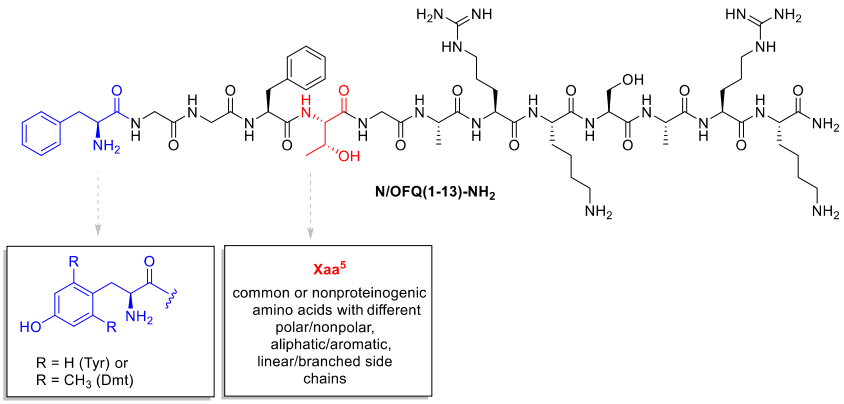

Figure 1. SAR investigation leading to a series of [Tyr/Dmt[1],Xaa[5]]N/OFQ(1-13)-NH_2 peptide derivatives as possible mixed NOP/opioid receptor ligands.

Table 1. Effects of Standard Agonists and a First Series of [Tyr[1],Xaa[5]]N/OFQ(1-13)-NH_2 Derivatives at NOP and mu Opioid Receptors in Calcium Mobilization Studies

		NOP		mu		NOP/mu
		pEC_{50} ($CL_{95\%}$)	E_{max} ± S.E.M.	pEC_{50} ($CL_{95\%}$)	E_{max} ± S.E.M	CR
1	N/OFQ(1-13)NH_2	9.29 (9.12−9.46)	368 ± 11	inactive		<0.001
2	dermorphin	inactive		7.71 (7.35−8.07)	380 ± 15	>50
3	[Tyr[1]]N/OFQ(1-13)-NH_2	9.23 (9.07−9.39)	380 ± 11	crc incomplete; 10 μM: 197 ± 69		0.001
4	[Tyr[1],Asn[5]]N/OFQ(1-13)-NH_2	8.70 (8.26−9.11)	327 ± 15	crc incomplete; 10 μM: 209 ± 29		0.002
5	[Tyr[1],Val[5]]N/OFQ(1-13)-NH_2	8.70 (8.53−8.87)	334 ± 19	crc incomplete; 10 μM: 254 ± 19		0.002
6	[Tyr[1],Lys(Ac)[5]]N/OFQ(1-13)-NH_2	8.55 (7.88−9.22)	370 ± 16	crc incomplete; 10 μM: 161 ± 31		0.003
7	[Tyr[1],Abu[5]]N/OFQ(1-13)-NH_2	8.15 (7.61−8.69)	366 ± 20	crc incomplete; 10 μM: 213 ± 37		0.007
8	[Tyr[1],Lys[5]]N/OFQ(1-13)-NH_2	7.26 (6.74−7.78)	366 ± 6	crc incomplete; 10 μM: 113 ± 35		0.05
9	[Tyr[1],Dap[5]]N/OFQ(1-13)-NH_2	7.20 (6.95−7.45)	367 ± 9	crc incomplete; 10 μM: 144 ± 44		0.06
10	[Tyr[1],Dab[5]]N/OFQ(1-13)-NH_2	6.43 (6.28−6.58)	340 ± 22	crc incomplete; 10 μM: 41 ± 4		0.37
11	[Tyr[1],Leu[5]]N/OFQ(1-13)-NH_2	8.03 (7.46−8.60)	339 ± 24	6.08 (5.95−6.21)	339 ± 25	0.01
12	[Tyr[1],Nle[5]]N/OFQ(1-13)-NH_2	8.77 (8.45−9.09)	375 ± 9	6.74 (6.15−7.33)	323 ± 4	0.01
13	[Tyr[1],Nva[5]]N/OFQ(1-13)-NH_2	8.43 (7.55−9.31)	358 ± 14	6.25 (5.59−6.91)	313 ± 19	0.007
14	[Tyr[1,5]]N/OFQ(1-13)-NH_2	7.07 (6.68−7.46)	332 ± 19	6.76 (6.34−7.18)	357 ± 21	0.49

tools for the characterization of delta and kappa-opioid receptor phenotypes.[18]

With the aim of generating a peptide acting as a nonselective NOP/opioid agonist, we investigated different approaches. On one hand, the peptide [Dmt[1]]N/OFQ(1-13)-NH_2 has been identified as a nonselective agonist for NOP and opioid receptors[19] and its tetrabranched derivative, generated using the peptide welding technology (PWT),[20] was demonstrated to produce a robust analgesic effect after spinal administration in nonhuman primates. However, this action was sensitive to NOP but not opioid receptor antagonists.[21] On the other hand, N/OFQ and dermorphin-related peptides were linked together to generate the hetero-tetrabranched derivative H-PWT1-N/OFQ-[Dmt[1]]dermorphin[22] or the dimeric compound DeNo.[23] Despite its promising *in vitro* pharmacological profile as a mixed NOP/opioid agonist, DeNo was not effective as a spinal analgesic.[23] In the present study, we further investigate the possibility of generating a mixed NOP/opioid agonist based on the following evidence: (i) mixed NOP/kappa ligands can be obtained combining the C-terminal sequence of N/OFQ with the N-terminal of dynorphin A, where amino acids in positions 5 and 6 were particularly important for receptor selectivity;[24] (ii) Thr[5] in N/OFQ(1-13)-NH_2 can be replaced with several different residues without loss of peptide efficacy and potency at the NOP receptor;[25] (iii) the substitution of Phe[1] in N/OFQ with Tyr[26] and particularly with Dmt[19,27,28] increases affinity/potency at classical opioid receptors. Thus, in the present study, 31 peptide derivatives with the general sequence [Tyr/Dmt[1],Xaa[5]]N/OFQ(1-13)-NH_2 were generated and tested for their action at NOP and opioid receptors (Figure 1).

Experimental data have been complemented by an *in silico* study of the binding of [Dmt[1,5]]N/OFQ(1-9)-NH_2 to the mu receptor. This non-natural peptide has been compared with the agonist peptide DAMGO ([D-Ala[2], *N*-MePhe[4], Gly-ol]-enkephalin) and the N-terminal fragment of N/OFQ (N/OFQ(1-9)-NH_2). The starting point of the computational study was the structure of the activated mu receptor in complex with the agonist peptide DAMGO that has been previously reported by X-ray diffraction and cryo-electron microscopy.[29,30] The last structure of the complex DAMGO-mu receptor was used as a model, allowing the setup of the two unknown complexes with the selected peptides by molecular docking. Specifically, docking of a flexible ligand to multiple receptor conformations as already applied to the study of NOP agonists and antagonists[31,32] was

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Table 2. Effects of Standard Agonists and [Tyr^1,Xaa^5]N/OFQ(1-13)-NH$_2$ Derivatives with Different Aromatic Residues as Xaa^5 at NOP and mu Opioid Receptors in Calcium Mobilization Studies

		NOP		mu		NOP/mu
		pEC$_{50}$ (CL$_{95\%}$)	E_{max} ± S.E.M.	pEC$_{50}$ (CL$_{95\%}$)	E_{max} ± S.E.M.	CR
1	N/OFQ(1-13)-NH$_2$	9.40 (9.19−9.61)	288 ± 15	inactive		<0.001
2	dermorphin	inactive		7.83 (7.56−8.11)	306 ± 23	>50
3	[Tyr^1]N/OFQ(1-13)-NH$_2$	9.13 (8.83−9.43)	266 ± 14	crc incomplete; 10 μM: 217 ± 29		0.001
15	[Tyr^1,Phe^5]N/OFQ(1-13)-NH$_2$	7.72 (7.56−7.87)	242 ± 11	6.41 (5.85−6.97)	315 ± 32	0.05
16	[Tyr^1,His^5]N/OFQ(1-13)-NH$_2$	7.39 (7.16−7.62)	249 ± 23	crc incomplete; 10 μM: 230 ± 37		0.05
17	[Tyr^1,Trp^5]N/OFQ(1-13)-NH$_2$	7.27 (7.19−7.35)	288 ± 28	crc incomplete; 10 μM: 193 ± 31		0.07
18	[Tyr^1,hPhe^5]N/OFQ(1-13)-NH$_2$	8.70 (8.24−9.16)	301 ± 26	crc incomplete; 10 μM: 228 ± 29		0.003
19	[Tyr^1,Phg^5]N/OFQ(1-13)-NH$_2$	7.57 (7.12−8.02)	293 ± 31	6.81 (6.33−7.29)	287 ± 48	0.17
20	[Tyr^1,p(OCH$_3$)Phe^5]N/OFQ(1-13)-NH$_2$	7.88 (7.81−7.95)	246 ± 18	crc incomplete; 10 μM: 221 ± 49		0.02
21	[Tyr^1,(pF)Phe^5]N/OFQ(1-13)-NH$_2$	7.31 (6.81−7.81)	311 ± 22	crc incomplete; 10 μM: 256 ± 55		0.06
22	[Tyr^1,(pNO$_2$)Phe^5]N/OFQ(1-13)-NH$_2$	7.31 (6.90−7.72)	305 ± 22	crc incomplete; 10 μM: 192 ± 64		0.06
23	[Tyr^1,Dip^5]N/OFQ(1-13)-NH$_2$	6.61 (6.13−7.09)	240 ± 26	6.78 (6.28−7.27)	331 ± 32	1.48
24	[Tyr^1,Bip^5]N/OFQ(1-13)-NH$_2$	6.93 (6.63−7.22)	272 ± 29	crc incomplete; 10 μM: 151 ± 66		0.15
25	[Tyr^1,1Nal^5]N/OFQ(1-13)-NH$_2$	7.17 (6.82−7.52)	245 ± 16	6.08 (5.67−6.49)	319 ± 31	0.08
26	[Tyr^1,2Nal^5]N/OFQ(1-13)-NH$_2$	6.82 (6.60−7.04)	266 ± 15	crc incomplete; 10 μM: 244 ± 10		0.19
27	[Tyr^1,(pNH$_2$)Phe^5]N/OFQ(1-13)-NH$_2$	7.52 (7.11−7.93)	274 ± 15	6.43 (5.80−7.05)	329 ± 37	0.08
28	[Tyr^1,Dmt^5]N/OFQ(1-13)-NH$_2$	7.75 (7.22−8.27)	251 ± 22	6.71 (6.36−7.07)	301 ± 35	0.09

Table 3. Effects of Standard Agonists and [Dmt^1,Xaa^5]N/OFQ(1-13)-NH$_2$ Derivatives at NOP and mu Opioid Receptors in Calcium Mobilization Studies

		NOP		mu		NOP/mu
		pEC$_{50}$ (CL$_{95\%}$)	E_{max} ± S.E.M.	pEC$_{50}$ (CL$_{95\%}$)	E_{max} ± S.E.M.	CR
1	N/OFQ(1-13)-NH$_2$	9.59 (9.30−9.88)	289 ± 34	inactive		<0.001
2	dermorphin	inactive		8.15 (7.97−8.33)	359 ± 18	>100
3	[Tyr^1]N/OFQ(1-13)-NH$_2$	9.15 (8.48−9.82)	236 ± 13	5.80 (5.22−6.38)	251 ± 34	<0.001
29	[Dmt^1]N/OFQ(1-13)-NH$_2$	8.57 (8.26−8.87)	294 ± 22	7.37 (7.12−7.51)	311 ± 20	0.06
30	[Dmt^1,Tyr^5]N/OFQ(1-13)-NH$_2$	6.91 (6.81−7.01)	304 ± 21	7.81 (7.47−8.15)	410 ± 25	7.94
31	[Dmt^1,Phe^5]N/OFQ(1-13)-NH$_2$	7.25 (6.69−7.81)	277 ± 28	8.19 (7.71−8.66)	335 ± 22	8.71
32	[Dmt^1,Phg^5]N/OFQ(1-13)-NH$_2$	6.95 (6.78−7.12)	282 ± 12	8.54 (8.13−8.96)	339 ± 28	39
33	[Dmt^1,1Nal^5]N/OFQ(1-13)-NH$_2$	7.22 (6.91−7.54)	286 ± 19	7.80 (7.51−8.09)	349 ± 20	3.80
34	[Dmt^1,(pNH$_2$)Phe^5]N/OFQ(1-13)-NH$_2$	7.58 (7.37−7.79)	258 ± 20	7.82 (7.35−8.29)	358 ± 15	1.73
35	[Dmt^{1,5}]N/OFQ(1-13)-NH$_2$	8.39 (8.05−8.72)	270 ± 25	8.08 (7.74−8.42)	354 ± 16	0.49

Table 4. Effects of Standard Agonists and [Dmt^{1,5}]N/OFQ(1-13)-NH$_2$ at NOP and mu Opioid Receptors in DMR Studies

		NOP		mu		mu/NOP
		pEC$_{50}$ (CL$_{95\%}$)	E_{max} ± S.E.M.	pEC$_{50}$ (CL$_{95\%}$)	E_{max} ± S.E.M.	CR
36	N/OFQ	9.37 (8.96−9.79)	209 ± 14	inactive		<0.001
2	dermorphin	inactive		8.92 (8.74−9.10)	151 ± 16	>500
35	[Dmt^{1,5}]N/OFQ(1-13)-NH$_2$	7.71 (6.65−8.76)	223 ± 11	8.64 (8.28−9.01)	202 ± 20	8.51

carried out to provide the best binding pose of the two peptides [Dmt^{1,5}]N/OFQ(1-9)-NH$_2$ and N/OFQ(1-9)-NH$_2$. This docking procedure was further challenged by long-lasting molecular dynamics (MD) simulations and compared with an MD simulation of the DAMGO-mu receptor-G$_i$ protein complex to identify the key interactions necessary for a successful nonselective NOP/opioid agonist. Finally, considering that NOP receptor agonists have demonstrated antitussive effects *in vivo*[33−37] and that opioids are effective drugs currently in use to treat cough,[38] the most potent mixed NOP/opioid agonist has been evaluated *in vivo* for its antitussive effects in guinea pigs.

RESULTS

Chemistry. The peptide derivatives reported in Tables 1−4 were prepared through automated Fmoc/*t*Bu-based solid-phase peptide synthesis (SPPS) on a Rink amide MBHA resin. Commercially available protected amino acids were employed as synthetic precursors of the target peptides except for Fmoc-2′,6′-dimethyl-tyrosine (Fmoc-Dmt-OH) that was instead synthesized in analogy to an approach previously published by Wang *et al.*[39] (Scheme S1 of the Supporting Information). Specifically, H-Tyr-OH was first esterified to H-Tyr-OMe under standard conditions, and then, the phenolic hydroxyl was protected with a *tert*-butyldimethylsilyl ether moiety before the following coupling with picolinic acid. The latter function worked as a directing group for the subsequent Pd(OAc)$_2$-catalyzed C−H alkylation with CH$_3$I and K$_2$CO$_3$ allowing the simultaneous and regioselective introduction of two methyl groups at the ortho-positions of the aromatic ring. Then, full deprotection under strongly acidic conditions, followed by treatment with Fmoc-Cl, led to the desired 2′,6′-dimethyl

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tyrosine scaffold (detailed procedures and analytical characterizations of Fmoc-Dmt-OH and its precursors have been reported in the Supporting Information). The structures of other nonproteinogenic amino acids employed in this work have been depicted in Table S1.

***In Vitro* Structure–Activity Relationship.** N/OFQ(1-13)-NH_2 stimulated calcium mobilization with high potency and maximal effects in cells coexpressing NOP receptors and chimeric G proteins, while being inactive in cells expressing the mu opioid receptor. On the contrary, dermorphin stimulated calcium mobilization with high potency and maximal effects in mu expressing cells, while it was inactive in NOP cells (Table 1). The substitution of Phe^1 with Tyr as in [Tyr^1]N/OFQ(1-13)-NH_2 did not affect NOP potency while promoting a minor increase in mu potency. Thr^5 in [Tyr^1]N/OFQ(1-13)-NH_2 was replaced with a series of both proteinogenic and nonproteinogenic amino acids with different polar/nonpolar, aliphatic/aromatic, linear/branched side chains with the aim to explore the effect of several structural parameters on the biological activity. The substitution of Thr^5 with Asn, Val, Lys(Ac) caused a slight (<10-fold) reduction in NOP potency and no modification of mu potency. The same substitution with Abu, Lys, Dap, and Dab induced a larger loss (>10-fold) of NOP potency. The introduction in position 5 of Leu, Nle, and Nva promoted a moderate decrease in NOP potency associated with a significant increase in mu potency. A similar increase in mu potency was achieved with [$Tyr^{1,5}$]N/OFQ(1-13)-NH_2, which however displayed a larger decrease in NOP potency; thus, the NOP/mu concentration ratio for this peptide was near 1 (Table 1). None of the amino acid substitutions evaluated in Table 1 modified ligand efficacy at both NOP and mu receptors. Based on these results, aromatic residues were selected for further modifications of position 5 of [Tyr^1]N/OFQ(1-13)-NH_2.

As shown in Table 2, 14 compounds with an aromatic residue substituting Thr^5 in [Tyr^1]N/OFQ(1-13)-NH_2 were assayed in NOP and mu receptor expressing cells. The different amino acids did not modify ligand efficacy but produced different effects on NOP and mu potency. In particular, the NOP potency of these derivatives was in the range of 8.70−6.61, while the mu potency of these compounds was <6 with the exceptions of peptides substituted with Phe, Phg, 1Nal, (pNH_2)Phe, and Dmt (range 6.08−6.81). Then, for further investigation, we selected those sequences showing pEC_{50} values >7 for the NOP receptor and >6 for the mu receptor associated with an NOP/mu concentration ratio >0.05. These criteria were matched by [Tyr^1]N/OFQ(1-13)-NH_2 derivatives substituted in position 5 with Tyr, Phe, Phg, 1Nal, (pNH_2)Phe, and Dmt.

The third series of peptides was generated by substituting Tyr^1 with Dmt that is known to increase opioid receptor potency.[40] In fact, as shown in Table 3, [Dmt^1]N/OFQ(1-13)-NH_2 displayed a moderate (10-fold) decrease in NOP potency compared to [Tyr^1]N/OFQ(1-13)-NH_2 associated to a more pronounced increase (approx. 40-fold) in mu potency. The substitution of Thr^5 of [Dmt^1]N/OFQ(1-13)-NH_2 with the above-mentioned amino acids generated results similar to those obtained with [Tyr^1]N/OFQ(1-13)-NH_2, that is, a slight to moderate decrease in NOP potency associated to a large increase in mu potency. The most exciting result has been obtained with [$Dmt^{1,5}$]N/OFQ(1-13)-NH_2 that displayed similar and high potency at both NOP and mu receptors.

[$Dmt^{1,5}$]N/OFQ(1-13)-NH_2 was further evaluated in dynamic mass redistribution (DMR) experiments performed on CHO cells expressing the human NOP and mu receptors. As summarized in Table 4, N/OFQ elicited a concentration-dependent positive DMR signal in cells expressing the NOP receptor being inactive in mu expressing cells. Opposite results were obtained with dermorphin that behaves as a mu-selective agonist. [$Dmt^{1,5}$].N/OFQ(1-13)-NH_2 elicited a robust DMR response in both cell lines with similar maximal effects to standard agonists. [$Dmt^{1,5}$].N/OFQ(1-13)-NH_2 displayed nanomolar potency at both NOP and the mu receptor with a mu/NOP potency ratio of 8.51 (Table 4).

Finally, the agonist properties of [$Dmt^{1,5}$]N/OFQ(1-13)-NH_2 were evaluated at delta and kappa opioid receptors in calcium mobilization experiments. As shown in Figure 2,

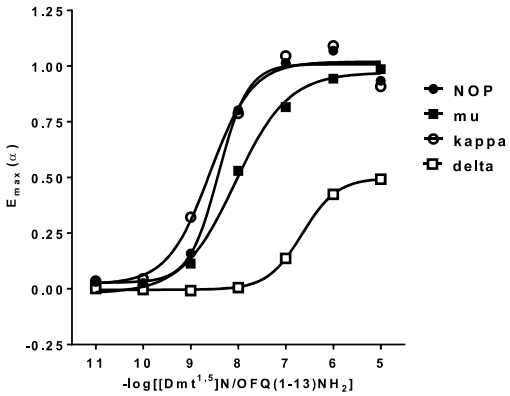

Figure 2. Effects of [$Dmt^{1,5}$]N/OFQ(1-13)-NH_2 at NOP and classical opioid receptors in calcium mobilization studies.

[$Dmt^{1,5}$]N/OFQ(1-13)-NH_2 displayed low potency and efficacy at the delta receptor. On the contrary, the peptide showed at the kappa opioid receptor high potency (pEC_{50} = 8.49) similar to that displayed at NOP (pEC_{50} = 8.39) and mu (pEC_{50} = 8.08) receptors. Thus, [$Dmt^{1,5}$]N/OFQ(1-13)-NH_2 should be classified as a mixed NOP/mu/kappa full agonist.

Molecular Dynamics. As explained in the Experimental Section, MD simulations have been performed setting up nine-residue long peptides (i.e., [Phe/Dmt^1,Thr/Dmt^5]N/OFQ(1-9)-NH_2) due to the fact that longer peptides lack reliable starting conformations by molecular docking. Moreover, in the following, we will focus on the first five residues of the peptides, those entering the mu opioid receptor orthosteric site, as residues 6−9 represent the more flexible part of the peptides along the MD simulation. The results obtained for [$Dmt^{1,5}$]N/OFQ(1-9)-NH_2 were compared with those obtained by similar simulations performed on the mu agonist peptide DAMGO and also on N/OFQ(1-9)-NH_2 as a sort of negative control since this peptide lacks mu receptor affinity.[26]

In Figure 3A, the 3D conformation obtained after docking and MD for [$Dmt^{1,5}$]N/OFQ(1-9)-NH_2 (colored purple) is superimposed to the known one reported for DAMGO (colored yellow, PDB code 6DDF).[30] Subsequent panels (Figure 3B−E) show the main interactions relating to each of the three aromatic residues, that is, Dmt^1, Phe^4, and Dmt^5. Furthermore, a general comparison between the results of MD simulations performed on DAMGO (blue), [$Dmt^{1,5}$]N/OFQ(1-9)-NH_2 (green), and N/OFQ(1-9)-NH_2 (red) is shown in Figure 4. In this figure, patterns of the main receptor-peptide interactions provided are

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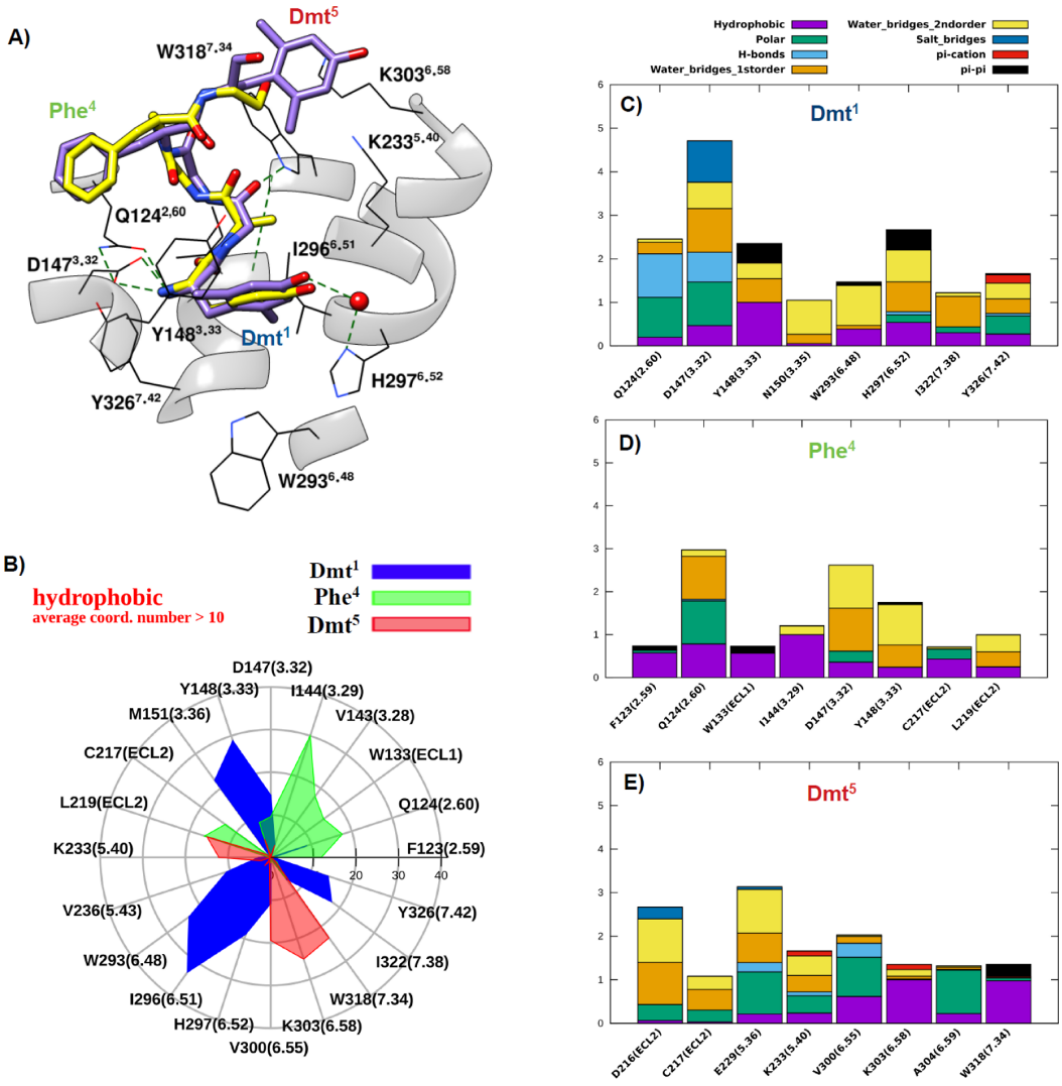

Figure 3. (A) Orthosteric site of [Dmt^{1,5}]N/OFQ(1-9)-NH_2 (colored purple) in the active mu receptor, according to "*in silico*" docking and MD (starting receptor structure from PDB code 6DDF). Only the first five residues are shown. The reported DAMGO conformation (the same PDB code) is superimposed (yellow). (B) Hydrophobic contacts between Dmt^1, Phe^4, and Dmt^5 with their neighboring residues. (C–E) Interaction histograms of residues Dmt^1, Phe^4, and Dmt^5, respectively, including hydrophobic, polar, H-bonds, water bridges of first and second order, salt bridges, and π–cation and π–π stacking as derived from long-lasting MD.

displayed and superimposed, that is, hydrophobic and polar average number of contacts (Figure 4A,B), percentage of formation of hydrogen bonds, and average "strength" of π–π stacking and π–cation interaction (Figure 4C–E, respectively). Accordingly, the representative conformation of [Dmt^{1,5}]N/OFQ(1-9)-NH_2 in the orthosteric site largely overlaps with that of DAMGO (Figure 3A). The N-terminus of Dmt^1 forms salt bridge/hydrogen bond contacts with D^{147} (a residue conserved all along the opioid family) similar to both DAMGO and the morphinan agonist BU72 (PDB code 5C1M).[29] MD simulations show that this important interaction is strongly stabilized by the presence of another conserved residue, Q^{124} (TM2), whose nitrogen and oxygen side-chain atoms reinforce the hydrogen bond network by contacts with both the carboxyl oxygen of D^{147} and the N-terminus of Dmt^1. Moreover, water bridges fill the small remaining volume between the D^{147} and Q^{124} side chains and the backbone donor/acceptors of Dmt^1, Gly^3, and Gly^2, with the latter being in direct H-bond with W^{318} of TM7.

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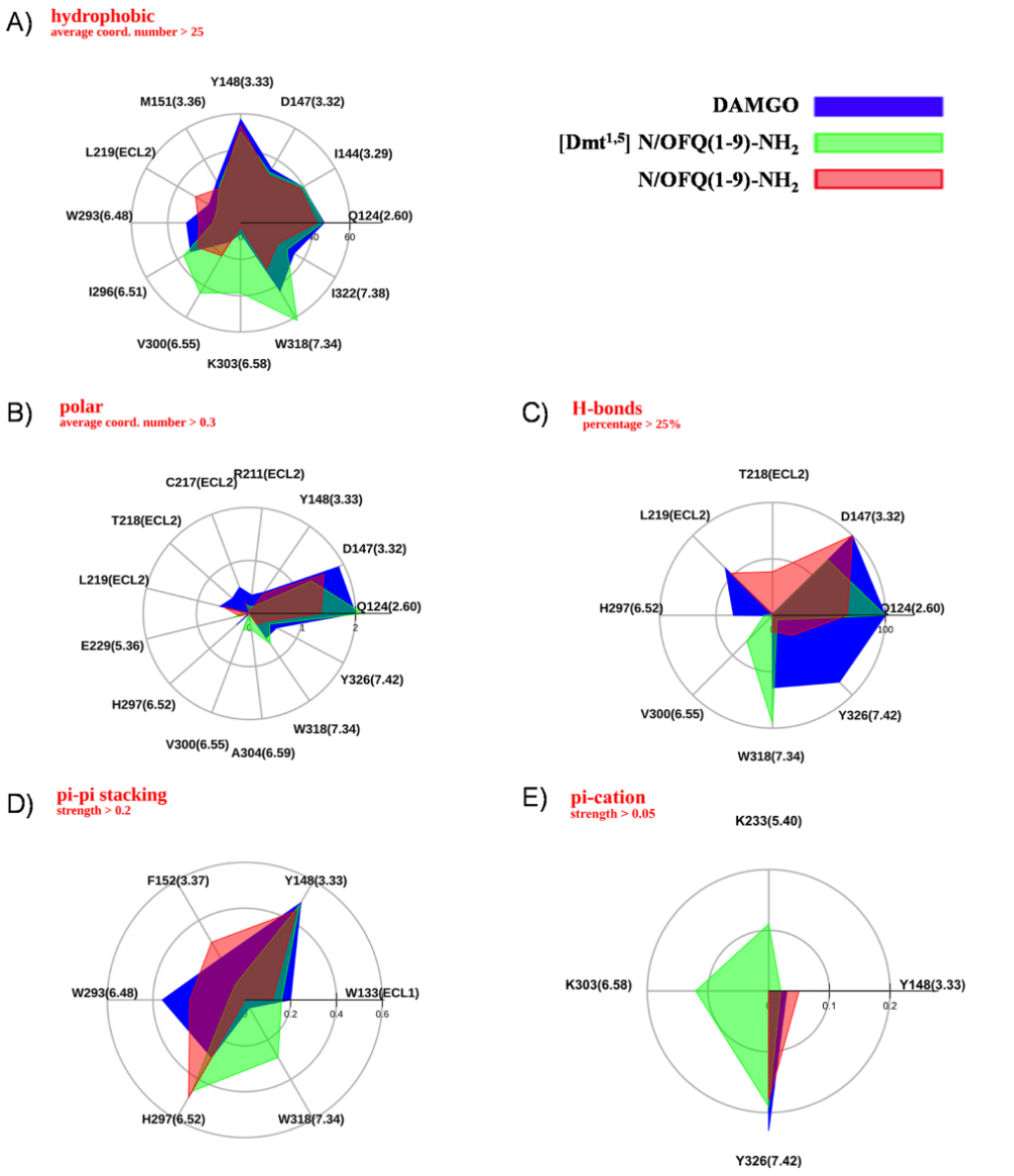

Figure 4. Maps of hydrophobic (A), polar (B), H-bond (C), $\pi-\pi$ stacking (D), and $\pi-$cation (E) interactions between the mu receptor and the studied ligands along MD trajectories. Only residues 1−5 are considered for [$Dmt^{1,5}$]N/OFQ(1-9)-NH_2 and N/OFQ(1-9)-NH_2.

Dmt^1 is also in direct hydrophobic contact with TM6 residues (W^{293}, H^{297}, and especially I^{296}, Figure 3B,C). Along the MD trajectories, its aromatic head moves alternating first- and second-order water bridges with H^{297} of TM6 (Figure 3C). Partial $\pi-\pi$ stacking between the Dmt^1 and H297 rings is also observed during the simulations. Hydrophobic, π-stacking, and water bridge contacts between Dmt^1 and Y^{148} (TM3), H^{297} and W^{293} (TM6) frequently occur (Figure 3C). The latter residue, in the so-called receptor polar cavity, is thought to be very important for the activation mechanism in many class A GPCRs, and these interactions, although not fully stable, could contribute to stabilize the receptor active state.

The formation of alternating second-order water bridges (along 78% of the trajectory) shows that the N-terminus of Dmt^1, together with D^{147}, is also in contact with N^{150} (Figure 3C), an important conserved residue that in the reported high-

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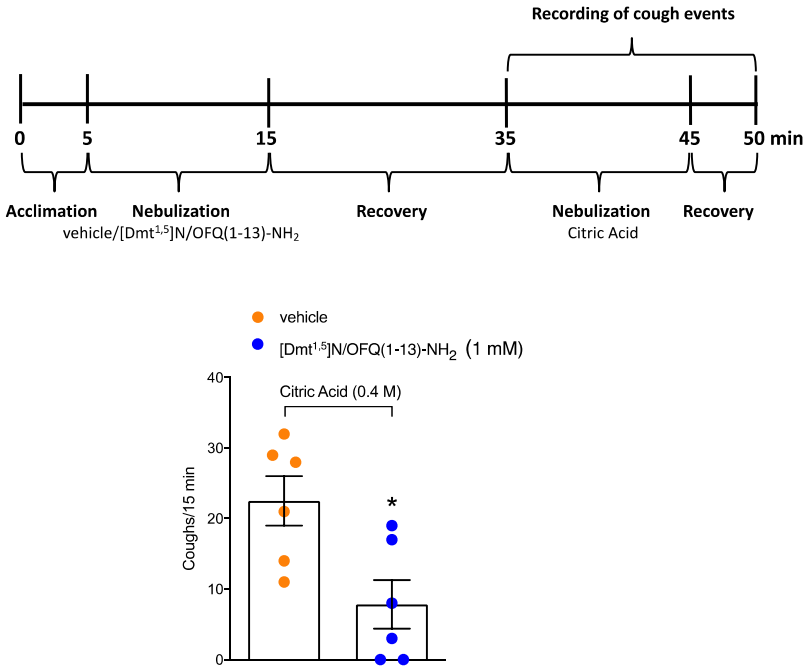

Figure 5. Effect of [$Dmt^{1,5}$]N/OFQ(1-13)-NH_2 on citric acid-induced cough in conscious guinea pigs. Schematic representation of the experimental procedure for the cough measurement in conscious guinea pigs and pooled data of cough number after [$Dmt^{1,5}$]N/OFQ(1-13)-NH_2 (1 mM) or vehicle (0.9% NaCl) nebulization, 30 min before the nebulization of the tussive agent, citric acid (0.4 M). Values are the mean ± SEM of the numbers of coughs/15 min, with data points overlaid (n = 6 guinea pigs for each condition). *$p < 0.05$ vs vehicle, Student's t-test.

resolution structure of the inactive delta receptor[41] is shown to connect the orthosteric site to the sodium pocket in the central part of the receptor.

While Dmt^1 interacts with both TM3 (more than 40 contacts with residues Y^{148} and M^{151}) and TM6 (about 80 contacts with residues W^{293}, I^{296}, and H^{297}), Phe^4 is immersed in the same hydrophobic pocket as the phenyl group of DAMGO between TM2 (residues F^{123} and Q^{124}) and TM3 (residues V^{143} and I^{144}) (Figure 3B,D), still participating with its amidic nitrogen and water bridges to the main hydrogen bond network linking the peptide to D^{147} and Q^{124} (Figure 3D).

The Dmt^5 peptide residue mainly interacts with residues not conserved within the opiate family, that is, E^{229} and K^{233} of TM5, V^{300}, and K^{303} of TM6, and W^{318} of TM7. Movements of this ring allow an alternation of nonpolar interactions with the aliphatic chains of K^{303} (TM6) and K^{233} (TM5) (Figure 3B) and of possible π−cation interactions with the positively charged amine of both the same K residues (Figure 3E). Similarly, the amidic oxygens of Gly^3, Dmt^5, and Gly^6 alternate in H-bond or water bridge contacts with R^{211} (ECL2) and E^{229} (TM5) on two opposite sides of the receptor.

Details on the MD simulations of DAMGO and [$Dmt^{1,5}$]N/OFQ(1-9)-NH_2 in complex with the mu receptor are given in Figures S1 and S2, reporting the root-mean-square deviation (RMSD) analyses and clustering outcomes for each of the investigated peptides. Moreover, the RMSD analysis (Figure S3) and the representative conformation of residues 1−5 of N/OFQ(1-9)-NH_2 (purple) are shown, compared to DAMGO (yellow). MD shows that the interactions of N/OFQ(1-9)-NH_2 with TM6 are strongly diminished; in addition to the absence of polar contacts and the water density between residues 1−5 of the peptide and TM6, there are only about 20 nonpolar contacts (between Phe^1 and W^{293} and between Phe^1 and F^{236}), while both polar and nonpolar interactions with TM3 increase.

In Figure 4, the maps of hydrophobic, polar, hydrogen bond, π−π stacking, and π−cation interactions for the peptides under study are superimposed for an overall immediate comparison. The hydrophobic and polar interaction maps are widely superimposable on all peptides (Figure 4A,B), attesting the similarity of their conformation inside the orthosteric site, with an increase of nonpolar contacts between [$Dmt^{1,5}$]N/OFQ(1-9)-NH_2 and TM6 (I^{296}, V^{300}, and K^{303}) essentially due to the aromatic ring of Dmt^5. The N-terminus of all three peptides forms hydrogen bonds with D^{147} and Q^{124} (Figure 4C). More interestingly, according to our simulation, the H-bond contact reported in the crystal structure between the amidic oxygen of Gly^3 of DAMGO and the indole nitrogen of W^{318} is not fully stable; in the same time, the phenolic head of Tyr^1 tends to extend toward the so-called "polar cavity" between Y^{326} and W^{293} in the intracellular side (Figure S1C), with the possibility to form π-stacking with W^{293} (Figure 4D) and a H-bond besides a π−cation contact between its N-terminus and the phenol group of Y^{326}. On the other hand, the H-bond between Gly^2 of [$Dmt^{1,5}$]N/OFQ(1-9)-NH_2 and W^{318} remains quite stable, as reinforced by partial π-stacking between W^{318} and Dmt^5 (Figures 3E and 4D), while the Dmt^1 phenolic head, sterically hindered by the two methyl groups, does not extend toward the polar cavity. Concerning N/OFQ (1-9)-NH_2, Phe^1 has

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negligible hydrogen and water bond contacts with the inner side of the receptor, and the contacts between Phe^4, Thr^5 of the peptide and T^{218}, L^{219} of extracellular loop 2 (ECL2) are stronger (Figure 4C). The N-terminus of the three peptides can form π−cation interactions with the aromatic ring of Y^{326}, while as mentioned above, π−cation contributions due to interactions of K^{233} and K^{303} are exclusive of [$Dmt^{1,5}$]N/OFQ(1-9)-NH_2 (Figure 4E).

***In Vivo* Experiments: [$Dmt^{1,5}$]N/OFQ(1-13)-NH_2 Effect on Citric Acid-Induced Cough in the Conscious Guinea Pig.** To test the antitussive effect of [$Dmt^{1,5}$]N/OFQ(1-13)-NH_2, we used a model of cough induced by citric acid in guinea pigs. Data showed that the coadministration of [$Dmt^{1,5}$]N/OFQ(1-13)-NH_2 with citric acid did not affect the tussive response (coughs/15 min: vehicle = 17.83 ± 3.95 vs [$Dmt^{1,5}$]N/OFQ(1-13)-NH_2 17.67 ± 1.73). However, the nebulization with [$Dmt^{1,5}$]N/OFQ(1-13)-NH_2 before (30 min) the challenge with the tussive agent significantly reduced the cough number induced by citric acid (Figure 5).

DISCUSSION

This structure activity investigation was aimed at the identification of novel peptides acting as mixed NOP/mu receptor agonists. To this aim, we substituted Phe^1 of N/OFQ(1-13)-NH_2 with amino acids containing a phenol moiety and Thr^5 with several proteinogenic and nonproteinogenic residues. Novel peptides were investigated in calcium mobilization experiments performed in cells expressing the human recombinant receptors and chimeric G proteins. The structure activity investigation led to the identification of the potent mixed agonist [$Dmt^{1,5}$]N/OFQ(1-13)-NH_2 whose NOP and mu agonist properties were confirmed in DMR studies. Moreover, [$Dmt^{1,5}$]N/OFQ(1-13)-NH_2 was also able to potently stimulate kappa but not delta opioid receptors. The capability of this peptide to bind the mu receptor has been also investigated in MD studies that suggested a similar active conformation for [$Dmt^{1,5}$]N/OFQ(1-13)-NH_2 and DAMGO and crucial interactions with D^{147} and H^{297}. Moreover, the binding of [$Dmt^{1,5}$]N/OFQ(1-13)-NH_2 is reinforced by additional polar interactions of Dmt^5 with K^{223} and K^{303}. Finally, [$Dmt^{1,5}$]N/OFQ(1-13)-NH_2 elicited a robust antitussive action *in vivo* in a model of cough induced by nebulization of citric acid in conscious guinea pigs.

Previous studies demonstrated that the substitution of Phe^1 in N/OFQ with Tyr reduces NOP selectivity over opioid receptors[26,42] and that Thr^5 of N/OFQ(1-13)-NH_2 can be substituted with different amino acids with no changes in peptide efficacy and relatively little modifications of potency.[25] Thus, we selected a series of amino acids to substitute Thr^5 in [Tyr^1]N/OFQ(1-13)-NH_2 in order to increase the mu receptor activity of the peptide derivatives. The results obtained with [Tyr^1]N/OFQ(1-13)-NH_2 derivatives were similar to those previously obtained with N/OFQ(1-13)-NH_2 derivatives in terms of NOP receptor activity. As far as the mu receptor is concerned, an increase in potency has been obtained with Leu, Nle, Nva, and Tyr. These results are not unexpected since Leu in position 5 is found in naturally occurring opioid ligands (Leu-enkephalin and dynorphin) and Nle (and possibly Nva) may mimic methionine, which is also present in position 5 of other endogenous opioid peptides (Met-enkephalin, beta-endorphin). In addition, the same can be said for Tyr^5, which is found in amphibian opioid peptides such as the mu-selective agonist dermorphin. Moreover, previous studies demonstrated that position 5 of enkephalin can be replaced with aromatic residues[43] or non-natural aliphatic residues[44] with no major changes of bioactivity. Interestingly, [$Tyr^{1,5}$]N/OFQ(1-13)-NH_2 displayed very similar potency at NOP and the mu opioid receptor; thus, with the aim to identify potent mixed NOP/mu agonists, further studies were performed substituting position 5 with aromatic amino acids.

Despite the investigation of 14 chemically different aromatic residues, no clear structure activity information was obtained. In fact, with the exception of $hPhe^5$, little changes in NOP potency were measured and the same can be said for mu receptor activity. Thus, for further studies, we selected compounds matching the following criteria: pEC_{50} > 7 for the NOP receptor and >6 for the mu receptor, with NOP/mu ratio > 0.05. This let us to select Tyr, Phe, Phg, 1Nal, (pNH_2)Phe, and Dmt to be substituted in position 5 of [Dmt^1]N/OFQ(1-13)-NH_2.

The opioid receptor binding enhancing properties of Dmt in position 1[19, 27, 28] were confirmed by the present results. In fact, compared to [Tyr^1]N/OFQ(1-13)-NH_2, [Dmt^1]N/OFQ(1-13)-NH_2 displayed approximately 3-fold reduced potency at NOP associated with almost 100-fold increased potency at the mu receptor. The same pattern of effects, that is, no change or modest reduction of NOP potency associated with a large increase in mu potency was obtained with [Dmt^1]N/OFQ(1-13)-NH_2 derivatives substituted in position 5 with Tyr, Phe, Phg, 1Nal, and (pNH_2)Phe. [$Dmt^{1,5}$]N/OFQ(1-13)-NH_2 is however the exception to this rule; in fact, this peptide displayed, compared to [Tyr^1,Dmt^5]N/OFQ(1-13)-NH_2, increased potency at both NOP and mu receptors. This led to an NOP/mu ratio of potency of [$Dmt^{1,5}$]N/OFQ(1-13)-NH_2 near 1. Interestingly, a very similar NOP/mu ratio was displayed by [$Tyr^{1,5}$]N/OFQ(1-13)-NH_2, which was however approximately 30-fold less potent at both receptors.

The calcium mobilization assay used in the present study has been previously set up[45,46] in our laboratories and then validated by investigating a large number of NOP and opioid receptor ligands.[19,23,47] However, this assay is based on the aberrant signaling generated by the expression of chimeric G proteins; therefore, we reassessed the pharmacological effects of [$Dmt^{1,5}$]N/OFQ(1-13)-NH_2 with the DMR assay. This test measures the physiological G_i-dependent signaling of NOP and opioid receptors as demonstrated by its sensitivity to pertussis toxin treatment.[48,49] DMR studies confirmed the mixed mu/NOP full agonist properties of [$Dmt^{1,5}$]N/OFQ(1-13)-NH_2.

Finally, the effects of [$Dmt^{1,5}$]N/OFQ(1-13)-NH_2 at kappa and delta opioid receptors were investigated. At delta receptors, [$Dmt^{1,5}$]N/OFQ(1-13)-NH_2 displayed low potency and efficacy, while it behaved as a potent full agonist at the kappa receptor. Of note, the kappa potency of [$Dmt^{1,5}$]N/OFQ(1-13)-NH_2 was similar to that shown at NOP and mu receptors. These results were not unexpected. In fact, binding experiments performed in guinea-pig brain membranes demonstrated the following rank order of affinity for [Tyr^1]N/OFQ(1-13)-NH_2: NOP > mu = kappa > delta.[26] Moreover, similar results have been previously obtained in functional studies performed with human recombinant receptors with [Dmt^1]N/OFQ(1-13)-NH_2 that displayed the following rank order of potency: NOP = mu > kappa > delta.[19] Collectively, these findings indicate that modifications of position 1 of N/OFQ such as Tyr and Dmt are sufficient for increasing mu and kappa but not delta receptor binding. Most probably, this is due to the fact that the C-terminal portion of N/OFQ is enriched in positively charged

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residues that may favor mu and kappa interactions but are detrimental for delta receptor binding.[50]

To get insights into the mechanisms by which [Dmt^{1,5}]N/OFQ(1-13)-NH_2 binds the mu receptor, MD studies were performed using the recently solved DAMGO-mu receptor-G_i complex.[30] The results obtained with [Dmt^{1,5}]N/OFQ(1-9)-NH_2 were compared with those of DAMGO and N/OFQ(1-9)-NH_2 used as the positive and negative control, respectively. These studies show that beyond the pivotal and expected interaction between the [Dmt^{1,5}]N/OFQ(1-9)-NH_2 N-terminus and D^{147}, the phenol oxygen of Dmt^1 can make first- or second-order water bridges with H^{297} (Gln in NOP) of TM6 of the mu receptor. This is in good agreement with the observation of a water bridge between the agonist BU72 and H^{297} in the active mu receptor and other small molecules or peptide mimetic agonists of kappa and delta receptors[30] and can account for the reduction of NOP selectivity and the increase of mu potency as simply induced by the presence of the phenol groups of Tyr^1 or Dmt^1 in N/OFQ instead of the phenyl group of Phe^1. Partial $\pi-\pi$ stacking between Dmt^1 (or Tyr^1) and H297 could further contribute to peptide stabilization in the orthosteric site, thus enhancing these effects. Analogous contacts have been reported for cocrystallized mu[51] and delta[52] but not NOP[53] antagonists. Importantly, Phe^1 of the N/OFQ sequence cannot form water bridges with H^{297}, and this is most probably the reason for the lack of mu affinity of the peptide.

Interestingly, as stated above, Dmt^5 mainly interacts with residues that differ within the opiate family, that is, E^{229} (G in NOP, D in kappa and delta) and K^{233} (A in NOP) of TM5, V^{300} (I in kappa), and K^{303} (W, E, and Q in delta, kappa, and NOP receptor, respectively) of TM6, and W^{318} (L in NOP) of TM7. While the carbonyl oxygen of Dmt^5 is in water bridge contact with E^{229}, its aromatic bulky head is stacked between the aliphatic chains of K^{303} and K^{233}, making possible $\pi-$cation interactions with the positively charged amine of both lysines (Figure 3B,E). In the reported crystal structure of mu-DAMGO[30] (PDB code 6DDF), the K^{303} positive charge is found at a 3.3 Å distance of the carbonyl oxygen of N(Me)-Phe of DAMGO, compatible with a weak H-bond, whereas K^{233} does not appear to contribute to the stabilization of the mu active state induced by both DAMGO and BU72;[29] the K^{233} amine group is found covalently linked to the antagonist β-funaltrexamine in the crystal structure of the inactive mu receptor.[51] As K^{303} and K^{233} are present in the mu but not the NOP receptor, the above-mentioned interactions between Dmt^5 and the two lysine residues could contribute to explain the mu-selective increase of affinity of [Dmt^{1,5}]N/OFQ(1-13)-NH_2 compared to [Dmt^1]N/OFQ(1-13)-NH_2, thus making [Dmt^{1,5}]N/OFQ(1-13)-NH_2 a mixed mu/NOP agonist. Last, as observed along the MD runs, the indole nitrogen of W^{318} in TM7 does not interact with N/OFQ but can form H-bond contact with Gly^2 of [Dmt^{1,5}]N/OFQ(1-9)-NH_2 as well as with DAMGO (49 and 63% of the trajectory, respectively). Thus, beyond differences in steric hindrance of Tyr^1 and Dmt^1 that may generically contribute to a larger hydrophobic core for the last one, the entity of the interaction between W^{318} and Gly^2 could also contribute to explain the enhanced potency of [Dmt^{1,5}] N/OFQ(1-13)-NH_2. Data obtained from this molecular modeling investigation are in agreement with those reported by recent studies performed on a series of cyclic opioid peptides.[54]

The role of the N/OFQ-NOP receptor system has been widely reported in several biological functions at the central and peripheral levels, including the cough reflex.[4] Previous studies showed that NOP receptor agonists given centrally or peripherally suppress capsaicin and acid inhalation-induced cough in guinea pigs.[33−37] Moreover, opioid drugs are widely used as antitussive agents,[38] and inhalation of encephalin was shown to be effective in reducing cough reflex *in vivo*.[55] The novel mixed NOP/opioid agonist [Dmt^{1,5}]N/OFQ(1-13)-NH_2 showed an inhibitory activity against citric acid-induced cough in guinea pigs, thus demonstrating the *in vivo* activity of the compound. However, further studies are needed to investigate the receptor mechanism involved in the antitussive action of the molecule.

CONCLUSIONS

In this study, starting from the NOP-selective sequence of N/OFQ(1-13)-NH_2, we developed a structure activity investigation focused on positions 1 and 5. Regarding position 1, a phenol moiety is required to increase mu receptor binding, and regarding position 5, aromatic residues generated the best results in terms of similar potency at NOP and mu receptors. This study led to the identification of [Dmt^{1,5}]N/OFQ(1-13)-NH_2 as the most potent mixed peptide agonist for NOP and mu receptors so far described in the literature. MD studies shed light on the molecular mechanisms adopted by this peptide to bind the active form of the mu receptor: some features of the mode of binding of [Dmt^{1,5}]N/OFQ(1-9)-NH_2 are superimposable to those of DAMGO, that is, the ionic bond with D^{147} of TM3 and the H-bond network with H^{297} of TM6, while others are peculiar of [Dmt^{1,5}]N/OFQ(1-9)-NH_2, that is, polar interactions of Dmt^5 with K^{223} and K^{303} of TM5 and TM6, respectively.

[Dmt^{1,5}]N/OFQ(1-13)-NH_2 is a novel mixed agonist for NOP and mu receptors that exerted antitussive effects in an *in vivo* model of cough. The compound will be evaluated in future studies for its antinociceptive properties. In fact, mixed NOP/mu agonists of both peptide and nonpeptide structures have been consistently demonstrated in preclinical studies to promote antinociceptive effects similar to those of morphine being however better tolerated particularly in terms of respiratory depression, tolerance, and abuse liability.[13] Importantly, phase II and III clinical studies performed with the mixed NOP/mu agonist cebranopadol have confirmed this favorable profile in pain patients.[9,56] Nowadays, the availability of safer analgesic drugs is particularly needed for facing the opioid epidemic that leads to a progressive increase of fatal overdoses over the past 2 decades.[57]

EXPERIMENTAL SECTION

Chemistry. *Materials and Methods.* All solvents and reagents were purchased from Sigma-Aldrich and Fisher Scientific. Enantiopure Fmoc-protected amino acids and the resins for SPPS were purchased from AAPPTec. Peptides were synthesized using a standard Fmoc/*t*-butyl strategy[58] with a Syro XP multiple peptide synthesizer (MultiSynTech GmbH, Witten Germany) on a Rink amide MBHA resin (4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxyacetamido-norleucyl-MBHA resin; loading 0.55 mmol/g). Fmoc-amino acids were used with a 4-fold excess on a 0.11 mM scale of the resin and coupled to the growing peptide chain using *N*,*N*′-diisopropylcarbodiimide and 1-hydroxybenzotriazole (DIC/HOBt, 4-fold excess) for 1 h at room temperature. Each Fmoc removal step was performed using 40% piperidine in *N*,*N*-dimethylformamide, and all the subsequent couplings were repeated until the desired peptide-bound resin was completed. The cleavage cocktail to obtain the peptides from the resin consisted of 95% trifluoroacetic acid, 2.5% water, and 2.5% triethylsilane, and cleavages were conducted for 3 h at room temperature. After filtration of the resin, diethyl ether was added to

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the filtrate to promote precipitation of the peptide products that were finally isolated by centrifugation. Reverse-phase purification of crude peptides was carried out on a Waters Prep 600 high-performance liquid chromatography (HPLC) system with a Jupiter column C18 (250 × 30 mm, 300 Å, 15 μm spherical particle size) using a gradient, programmed time by time, of acetonitrile/water [with 0.1% trifluoroacetic acid (TFA)] at a flow rate of 20 mL/min. Nonpeptide derivatives were purified through flash column chromatography using a Biotage System Isolera One. Analytical HPLC was performed with a Beckman 116 liquid chromatograph furnished of a UV detector. The purity of peptides in Table 1 was assessed with a Symmetry C18 column (4.6 × 75 mm, 3.5 μm particle size, SYSTEM GOLD) at a flow rate of 0.5 mL/min using a linear gradient from 100% of A (water + 0.1% TFA) to 100% of B (acetonitrile + 0.1% TFA) over a period of 25 min. The purity of peptides in Tables 2 and 3 was assessed with an Agilent Zorbax C18 column (4.6 × 150 mm, 3.5 μm particle size, KARAT32) at a flow rate of 0.7 mL/min using a linear gradient from 100% of A (water + 0.1% TFA) to 100% of B (acetonitrile + 0.1% TFA) over a period of 25 min. All final compounds were monitored at 220 nm showing ≥95% purity, and their molecular weights were confirmed using an ESI Micromass ZQ, Waters (HPLC chromatograms and ESI mass spectra of the final peptide derivatives have been reported in the Supporting Information). ^{1}H and ^{13}C NMR spectra were recorded for nonpeptide derivatives on a Varian 400 MHz instrument, and all experiments were performed in deuterated DMSO using its residual shifts as reference (s: singolet, d: doublet, dd: double doublet, t: triplet, m: multiplet).

***In Vitro* Pharmacological Studies.** *Drugs and Reagents.* [D-Pen^{2},D-Pen^{5}]enkephalin (DPDPE) and naltrexone were purchased from Tocris Bioscience (Bristol, UK). Concentrated solutions (1 mM) were made in bidistilled water and kept at −20 °C until use. The medium and reagents for cell culture were from Euroclone (Milan, Italy). Fluo-4 AM and pluronic acid were from Invitrogen/Thermo-Fisher Scientific (Waltham, USA). *N*-(2-Hydroxyethyl)piperazine-*N*′-ethanesulfonic acid (HEPES), probenecid, brilliant black, and bovine serum albumin (BSA) fraction V were from Sigma-Aldrich (St. Louis, USA).

Calcium Mobilization Assay. CHO cells stably coexpressing the human NOP or kappa or the mu receptor and the C-terminally modified $G_{\alpha qi5}$ and CHO cells coexpressing the delta receptor and the $G_{\alpha qG66Di5}$ protein were generated and cultured as described previously.[45,46] Cells were maintained in Dulbecco's modified Eagle's medium/nutrient mixture F-12 (DEMEM/F-12) supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin, 100 μg/mL hygromicin B, and 200 μg/mL G418 and cultured at 37 °C in 5% CO_2 humidified air. Cells were seeded at a density of 50,000 cells/well into 96-well black, clear-bottom plates. The following day, the cells were incubated with Hanks' balanced salt solution (HBSS) supplemented with 2.5 mM probenecid, 3 μM of the calcium-sensitive fluorescent dye Fluo-4 AM, and 0.01% pluronic acid for 30 min at 37 °C. After that time, the loading solution was aspirated and 100 μL/well of HBSS supplemented with 20 mM HEPES, 2.5 mM probenecid, and 500 μM brilliant black was added. Serial dilutions were carried out in HBSS/HEPES (20 mM) buffer (containing 0.02% BSA fraction V). After placing both plates (cell culture and master plate) into the fluorometric imaging plate reader FlexStation II (Molecular Devices, Sunnyvale, CA), fluorescence changes were measured. On-line additions were carried out in a volume of 50 μL/well. To facilitate drug diffusion into the wells, the present studies were performed at 37 °C. Maximum change in fluorescence, expressed as percent over the baseline fluorescence, was used to determine agonist response.

DMR Assay. CHO cells stably expressing the human NOP and mu receptors were kindly provided by D.G. Lambert (University of Leicester, UK). Cells were cultured in DMEM/F-12 medium supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/L L-glutamine. The medium was supplemented with 400 μg/mL G418 to maintain expression. Cells were cultured at 37 °C in 5% CO_2 humidified air. For DMR measurements, the label-free EnSight Multimode Plate Reader (Perkin Elmer, MA, US) was used. Cells were seeded 15,000 cells/well in a volume of 30 μL onto fibronectin-coated 384-well DMR microplates and cultured for 20 h to obtain confluent monolayers. Cells were starved in the assay buffer (HBSS with 20 mM HEPES, 0.01% BSA fraction V) for 90 min before the test. Serial dilutions were made in the assay buffer. After reading the baseline, compounds were added in a volume of 10 μL; then, DMR changes were recorded for 60 min. Responses were described as picometer (pm) shifts over time (sec) following subtraction of values from vehicle-treated wells. Maximum picometer (pm) modification (peak) was used to generate concentration response curves. All the experiments were carried out at 37 °C.

Data Analysis and Terminology. The pharmacological terminology adopted in this paper is consistent with IUPHAR recommendations.[59] All data are expressed as the mean ± standard error of the mean (SEM) of at least three experiments performed in duplicate. For potency values, 95% confidence limits ($CL_{95\%}$) were indicated. Agonist potencies are given as pEC_{50}, that is, the negative logarithm to base 10 of the molar concentration of an agonist that produces 50% of the maximal effect of that agonist. Concentration-response curves to agonists were fitted to the classical four-parameter logistic nonlinear regression model:

$$\text{Effect} = \text{Baseline} + (E_{max} - \text{Baseline})/(1 + 10^{(\text{LogEC}_{50} - \text{Log[compound]}) \times \text{Hillslope}})$$

Curve fitting was performed using PRISM 6.0 (GraphPad Software Inc., San Diego).

Molecular Dynamics. The setup of an *in silico* model of the non-natural peptides [Dmt^{1,5}] N/OFQ(1-9)-NH_2 and N/OFQ(1-9)-NH_2 in complex with the human mu receptor has been described in the Supporting Information. Classical MD simulations of these two receptor-peptide complexes were performed and compared with an MD simulation of the experimental system DAMGO-mu receptor-G_i protein complex as derived by the PDB file 6DDF.[30] The GROMACS 2018.3 package[60] was used under the AMBER parm99sb force field[61] at the full atomistic level using a TIP3P water solvent and an explicit pre-equilibrated phospholipid bilayer of 128 POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine) molecules obtained by the Prof. Tieleman website (http://moose.bio.ucalgary.ca). All the MD sessions were performed in a water−membrane system prepared as previously described.[31,32] The receptor-peptide-membrane systems were solvated in a triclinic water box (having basis vector lengths of 7, 7.4, and 9.3 nm) under periodic boundary conditions for a total number of about 45,000 atoms (6400 solvent molecules). The total charge of the system was neutralized by randomly substituting water molecules with Na^{+} ions and Cl^{-} ions to obtain neutrality with a 0.15M salt concentration. Following a steepest descent minimization algorithm, the system was equilibrated under canonical ensemble (*NVT*) conditions for 300 ps using a V-rescale, modified Berendsen thermostat with position restrains for both the receptor-peptide complex and the lipids and thereafter in a isothermal−isobaric ensemble (*NPT*) for 500 ps, applying position restraints to the heavy atoms of the protein-peptide complex, and using a Nose−Hoover thermostat and a Parrinello-Rahman barostat at 1 atm with a relaxation time of 2.0 ps. The MD simulation of the mu receptor-DAMGO-G_i protein was carried out on the whole ternary complex without positional restraints. On the other hand, in order to reduce the computational time, in the two mu receptor-peptide complexes, the G_i protein was not included in the system, but all residues within 5 Å of the G_i protein interface were restrained to the initial structure of the activated receptor using 5.0 kcal mol^{-1} $Å^{-2}$ harmonic restraints applied to non-hydrogen atoms. Using such restraints ensures that the receptor maintains an active conformation throughout the simulation. MD runs were performed under *NPT* conditions at 300 K with a T-coupling constant of 1 ps. van der Waals interactions were modeled using a 6−12 Lennard-Jones potential with a 1.2 nm cutoff. Long-range electrostatic interactions were calculated, with a cutoff for the real space term of 1.2 nm. All covalent bonds were constrained using the LINCS algorithm. The time step employed was 2 fs, and the coordinates were saved every 5 ps for analysis.

The MD analysis of the DAMGO-mu receptor-G_i protein complex (Figure S1) shows an overall stability of the starting configuration (corresponding to the crystal structure) with some motion of the phenolic head toward the intracellular side of the receptor, still conserving the water bridge contact with H^{297}. A non-negligible rearrangement is observed (Figures S2 and S3) along the MD sessions,

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starting from the docked conformations of [Dmt^{1,5}]N/OFQ(1-9)-NH_2 and N/OFQ(1-9)-NH_2, probably due to the limitations of the docking procedures applied to molecules with a large number of torsions, and confirms the importance of performing long-lasting MD sessions. Analysis of MD trajectories was performed using state-of-the-art computational tools, as described in the Supporting Information.

Artwork. 3D images of peptide-receptor structures were obtained by the Chimera software.[62]

***In Vivo* Pharmacological Studies.** *Animals.* Guinea pigs (Dunkin Hartley, male, 400−450 g, Charles River, Milan, Italy) were used. The group size of $n = 6$ animals was determined by sample size estimation using G*Power (v3.1)[63] to detect the size effect in a post-hoc test with type 1 and 2 error rates of 5 and 20%, respectively. Allocation concealment to the vehicle(s) or treatment group was performed using a randomization procedure (http://www.randomizer.org/). The assessors were blinded to the identity (allocation to the treatment group) of the animals. Guinea pigs were housed in a temperature- and humidity-controlled vivarium (12 h dark/light cycle, free access to food and water) for at least 1 week before the start of the experiments. Cough experiments were done in a quiet, temperature-controlled (20−22 °C) room between 9 am and 5 pm and were performed by an operator blinded to the treatment. All experiments were carried out according to the European Union (EU) guidelines for animal care procedures and the Italian legislation (DLgs 26/2014) application of the EU Directive 2010/63/EU. All animal studies were approved by the Animal Ethics Committee of the University of Florence and the Italian Ministry of Health (permit #450/2019-PR) and followed the animal research reporting *in vivo* experiment (ARRIVE) guidelines.

Measurement of Cough in Conscious Guinea Pigs. Cough experiments were performed using a whole-body plethysmography system (Buxco, Wilmington, NC, USA, upgraded version 2018).[64] The apparatus consists of four plethysmographs (four transparent Perspex chambers) ventilated with a constant airflow and each provided by a nebulizing head (Aerogen) and adjustable bias flow rates for acclimation and nebulization. The particle size presents an aerodynamic mass median diameter of 6 μm, and the output of the nebulizing heads can be set in the range between 0 and 0.4 mL per minute. The number of elicited coughs was automatically counted using the instrument. The nebulization rate used in the following experiments was 0.15 mL/min, and the air flows were 1750 mL/min during the acclimation phase and 800 mL/min during nebulization. These rates were previously found in our lab to elicit a significant number of cough events in the citric acid-induced cough model.

On the day of experiments, guinea pigs were individually placed into the chambers and let to acclimate for 10 min. To test the antitussive effect of [Dmt^{1,5}]N/OFQ(1-13)-NH_2, two different protocols were used. Protocol 1: after acclimation, a mixture of [Dmt^{1,5}]N/OFQ(1-13)-NH_2 (1 mM) or its vehicle (0.9% NaCl) and the tussive agent, citric acid (0.4 M), was nebulized for 10 min. During the 10 min of nebulization and for 5 min immediately post challenge (recovery period), the number of elicited coughs was automatically recorded using the BUXCO system. Protocol 2: after acclimation, [Dmt^{1,5}]N/OFQ(1-13)-NH_2 (1 mM) or its vehicle (0.9% NaCl) was nebulized for 10 min. After 20 min of recovery, the tussive agent, citric acid (0.4 M), was delivered by aerosol via a nebulizer for 10 min. During the 10 min of the citric acid challenge and 5 min immediately post challenge (recovery period), the number of elicited coughs was automatically recorded using the BUXCO system.

For the *in vivo* experiment, the statistical significance of differences between groups was assessed using Student's *t*-test.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jmedchem.0c02062.

Synthesis of Fmoc-2′,6′-dimethyltyrosine; synthetic procedures for the preparation of Fmoc-2′,6′-dimethyltyrosine; structures of nonproteinogenic amino acids; HPLC chromatograms and ESI mass spectra of the final peptide derivatives; model setup of non-natural peptides for MD; model setup of the mu receptor; model setup of the peptide-mu receptor complexes; and analysis of MD trajectories (PDF)

Molecular formula strings (CSV)

Model coordinates: representative structure of the [Dmt^{1,5}]N/OFQ(1-9)-NH_2-mu receptor complex (PDB)

Model coordinates: representative structure of the N/OFQ(1-9)NH_2-mu receptor complex (PDB)

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Notes

The authors declare the following competing financial interest(s): S.P., V.A., D.I., C.T., E.M., C.R., D.P., G.C., and R.G. are inventors of the patent application (102020000025972) focused on NOP/mu mixed agonists. G.C. and R.G. are founders of the University of Ferrara spin off company UFPeptides s.r.l., the assignee of such patent application. C.R. is CEO of UFPeptides s.r.l.

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■ ABBREVIATIONS

CHO cells, chinese hamster ovary cells; $CL_{95\%}$, 95% confidence limits; CR, concentration ratio; crc, concentration−response curve; DAMGO, [D-Ala^2, *N*-MePhe^4, Gly-ol]-enkephalin; DIC, *N*,*N*′-diisopropylcarbodiimide; DIPEA, *N*,*N*-diisopropylethylamine; DMEM/F-12, Dulbecco's modified Eagle's medium/nutrient mixture F-12; DMR, dynamic mass redistribution; DPDPE, [D-Pen^2,D-Pen^5]enkephalin; EtOAc, ethyl acetate; FBS, fetal bovine serum; FmocCl, Fmoc chloride; HATU, hexafluorophosphate azabenzotriazole tetramethyl uronium; HBSS, Hanks' balanced salt solution; LINCS, LINear Constraint Solver; MBHA resin, 4-methylbenzhydrylamine resin; N/OFQ, nociceptin/orphanin FQ; *NPT*, isothermal−isobaric ensemble; *NVT*, canonical ensemble conditions; PWT, peptide welding technology; SEM, standard error of the mean; SPPS, solid-phase peptide synthesis

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2.3 Pharmacological characterization of standard and novel TRPA1 antagonists

1. Introduction

The transient receptor potential (TRP) channel superfamily is the most represented family of receptors on primary sensitive terminations (Nilius and Szallasi 2014). At the level of primary afferent nerve fibers are expressed various types of ion channels that help to detect harmful stimuli in the environment, and amplify the transmission of nociceptive signals to central pain-relay neurons, causing pain sensation, and in pathophysiological conditions may contribute to sustaining chronic pain and prolonged suffering (Koivisto et al. 2018). One type of channel receptor that is expressed on nociceptors fibers is the TRP superfamily, which is able to detect and transduce various harmful stimuli (Koivisto et al. 2018). The most involved TRP receptor in pain signaling is the transient receptor potential vanilloid 1 (TRPV1) which is sensitive to capsaicin but is also activated by noxious heat. This receptor is often co-expressed with the transient receptor potential ankyrin 1 (TRPA1), which is a good target for pain relief (Koivisto et al. 2018).

In recent work, it was demonstrated that TRPA1 is co-expressed in a subpopulation of unmyelinated C-fibers with TRPV1, substance P, and CGRP. These latter compounds are released after activation of the C-fibers, conveying nociceptive signals to the brain and inflammation in tissues, eliciting meningeal vasodilatation and mediating neurogenic inflammation (De Logu et al. 2022). Moreover, TRPA1 is also expressed in Schwann cells, which seem to mediate the mechanical allodynia in a mouse model of neuropathic pain (De Logu et al. 2017, 2021). TRPA1 at the level of these cells seems to support the onset of migraine, particularly the onset of periorbital mechanical allodynia (PMA) (De Logu et al. 2022). Indeed, it was demonstrated that TRPA1 is sensitive to the redox state of the milieu, being activated by a wide series of reactive oxygen, nitrogen, and carbonyl species (ROS, RNS, and RCS), largely increased in the site of inflammation and after a tissue injury (Takahashi et al. 2011; Nassini et al. 2014; Kallenborn-Gerhardt et al. 2012). Moreover, De Logu and colleagues recently demonstrated that the CGRP injection in a mouse model of migraine, causes the release of nitric oxide (NO) and the consequent activation of TRPA1, at the level of Schwann cells. Its activation promotes the production of H_2O_2 Ca^{2+}-dependent, which sustains the activation mechanism of TRPA1 and the ROS release. That's corroborated by the evidence that mice with a selective deletion of TRPA1 in Schwann cells, or mice treated with selective TRPA1 antagonists, showed a markedly reduce PMA following CGRP injection (De Logu et al. 2022).

These findings added to the fact that a wide diversity of migraine triggers have been identified as TRPA1 activators (Nassini et al. 2012; Edelmayer et al. 2012), and a number of drugs used for migraine treatment desensitized or inhibit TRPA1 (Nassini et al. 2015;

Andersson et al. 2011; Nassini et al. 2010), make TRPA1 an interesting target for several painful pathologies. The goal of this work was the in vitro pharmacological characterization of standard and new TRPA1 antagonists, using the intracellular calcium mobilization assay. The novel TRPA1 antagonists designed and characterized in the frame of this study are analogs of the standard TRPA1 antagonist DHC200 (WO2010141805)

2. Materials and Methods

Drugs and reagents – Brilliant black, bovine serum albumin (BSA), 4-(2-hydroxyethyl)-1-piperazineethanesulforic acid (HEPES), and probenecid were from Sigma Aldrich (St. Louis, MO, USA). Pluronic acid and Fluo-4 AM were from Thermo Fisher Scientific (Waltham, US). All cells culture media and supplements were from Euroclone (Milano, Italy). The natural standards agonist cinnamaldehyde (CA), allyl isothiocyanate (AITC), benzyl isothiocyanate (BITC), the non-electrophilic compound PF-4840154, and the standards antagonists HC030031, A967079, DHC200, and all new compounds have been dissolved in dimethyl sulfoxide (DMSO, 10 mM). Stock solutions were kept at -20°C until use. Thirty-one new analogues of DHC200 were synthesized in the Department of Chemical, Pharmaceutical and Agricultural Sciences of the University of Ferrara (Italy), by Prof. D. Preti's research group. Stock solutions were made in DMSO (10 mM) and stored at -20 °C.

Cells – adenocarcinoma human alveolar basal epithelial cells (A549) were maintained in Dulbecco's Medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100µg/ml streptomycin, and 2mM L-glutamine. Cells were cultured at 37°C in 5% CO_2 humidified air.

Calcium mobilization assay – cells were seeded at a density of 45,000 cells/well in 100 µl in 96-well black, clear-bottom plates. The following day, cells were incubated with medium supplemented with 2.5 mM probenecid, 3 µM of the calcium-sensitive fluorescent dye Fluo-4 AM and 0.01% pluronic acid, for 30 min at 37 °C. After that time the loading solution was aspirated and 100 µl of HBSS supplemented with 20 mM HEPES, 2.5 mM probenecid, and 500 µM Brilliant Black were added. Cell culture and drug plates were placed into a fluorimetric imaging plate reader (FlexStation II, Molecular Devices, Sunnyvale, CA) and fluorescence changes were measured. On-line additions were carried out in a volume of 50 µl/well. To facilitate drug diffusion into the wells the present studies were performed at 37 °C. Additionally, in antagonism experiments, three cycles of mixing (25 µl from each well moved up and down 3 times) were performed immediately after agonism injection into the wells. In antagonism experiments standard antagonists and new compounds were injected into the wells 24 min before adding PF-4840154.

Data analysis and terminology – all data were analyzed using Graph Pad Prism 9.0 (La Jolla, CA, USA). Concentration-response curves were fitted using the four parameters log-logistic equation. Data are expressed as mean ± sem of *n* experiments performed in duplicate. Agonist potency was expressed as pEC_{50}, which is the negative logarithm to base 10 of the agonist molar concentration that produces 50% of the maximal possible effect of that agonist. Two types of experiments were performed to investigate the antagonist potency of

standard TRPA1 antagonists: i) inhibition-response curves to the antagonist against a fixed concentration of agonist (PF-4840154) approximately corresponding to its EC_{80}, ii) concentration-response curves to the agonist (PF-4840154) in the absence and in presence of a fixed concentration of antagonist.

The antagonist potency for the antagonist in inhibition-response curves experiments was expressed as pK_B, which was calculated as the negative logarithm to base 10 of the K_B from the following equation:

$$K_B = \left[\frac{IC_{50}}{([2 + (\frac{[A]}{EC_{50}})^n]^{1/n} - 1)} \right]$$

where IC_{50} is the concentration of antagonist that produces 50% inhibition of the agonist response, [A] is the concentration of agonist, EC_{50} is the concentration of agonist producing a 50% maximal response and n is the slope coefficient of the concentration-response curve to the agonist (Kenakin 2022).

When antagonists were assayed at a single concentration against the concentration-response curve to the agonist their potency was derived with the Gaddum Schild equation:

$$pA_2 = \log \left[\frac{CR - 1}{antagonist} \right]$$

assuming a slope value equal to unity, where CR indicates the ratio between agonist potency in the presence and absence of antagonist.

All new compounds were tested as agonists, for their ability to increase per se intracellular calcium levels. Inactive compounds were then tested as antagonists, by testing the concentration-response curves to PF-4840154 in the absence and in presence of a fixed concentration of antagonist; their potency (pA_2) was derived using the Gaddum Shild equation.

3. Results

Standard agonists – In the first series of experiments the agonists CA, BITC, AITC and PF-4840154 (figure 1) were tested for their effects on A549 cells in the calcium mobilization assay.

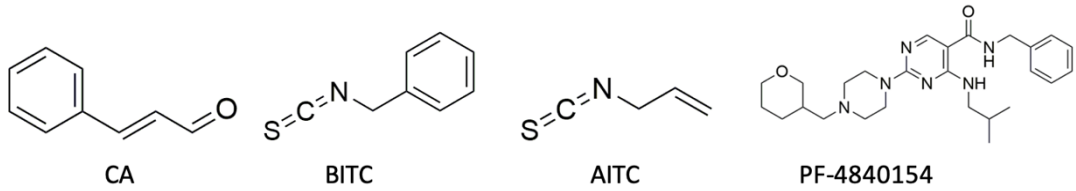

Figure 1. chemical structures of standard TRPA1 agonists

All compounds were able to stimulate the release of intracellular calcium in a concentration-dependent manner (figure 2). AITC, CA, BITC, and PF4840154 stimulated the release of intracellular calcium with potency values of 4.76, 4.57, 5.09, and 6.48, respectively. These data are summarized in table 1. Based on these results PF-4840154 was selected as a TRPA1 agonist for antagonism studies.

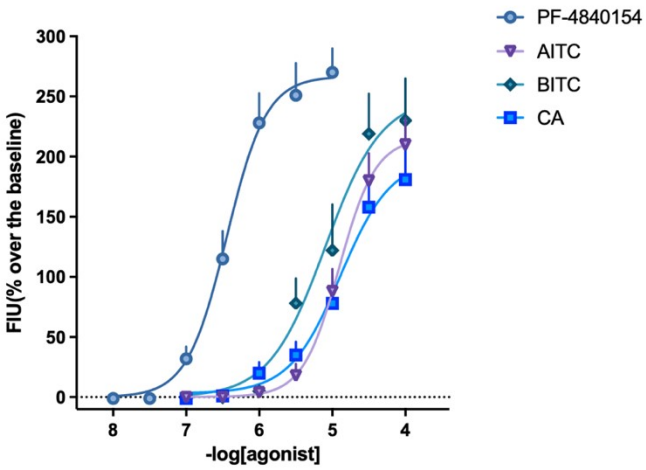

Figure 2. Calcium mobilization assay in A549 cells. Concentration-response curves to AITC, CA, BITC, and PF-4840154. Data are mean ± sem of at least 4 separate experiments made in duplicate.

Table 1. Potencies (pEC_{50}) and efficacy (E_{max}) of standard TRPA1 agonists

	pEC_{50} ($CL_{95\%}$)	$E_{max} \pm sem$
PF-4840154	6.48 (6.34 – 6.62)	270 ± 20%
AITC	4.92 (4.73 – 5.12)	210 ± 22%
BITC	5.03 (4.79 – 5.27)	230 ± 35%
CA	4.91 (4.68 – 5.14)	270 ± 20%

Standard antagonists –A967079, HC030031, and DHC200 (Figure 3) tested as agonists up to 100 µM (HC030031), and 10 µM (A967079 and DHC200) did not modify per se the intracellular calcium levels in A549 cells. In inhibition-response curve experiments, increasing concentrations of antagonists (0.1 µM – 100 µM for HC030031; 0.01 µM – 10 µM for A967079 and DHC200) were tested against 1 µM PF-4840154. A967079, HC030031, and DHC200, completely inhibited the effect of PF-4840154 in a concentration-dependent manner. The pK_B values estimated for these compounds are reported in table 2. Then, a fixed concentration of antagonist was tested against the concentration-response curves to PF-4840154. All reference antagonists were able to shift to the right the concentration-response curve to PF-4840154 (Figure 4). The pA_2 values calculated for these compounds were summarized in table 2.

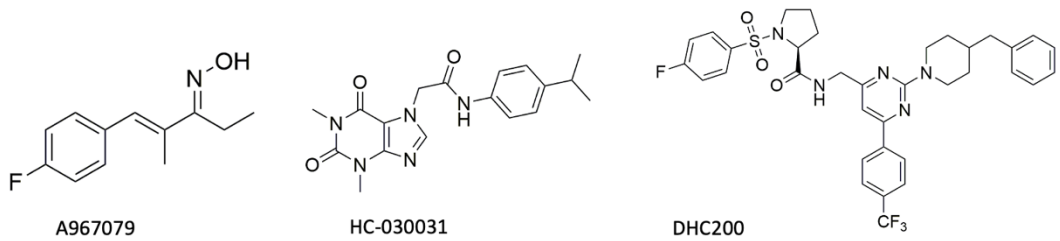

Figure 3. chemical structures of standard antagonists of TRPA1

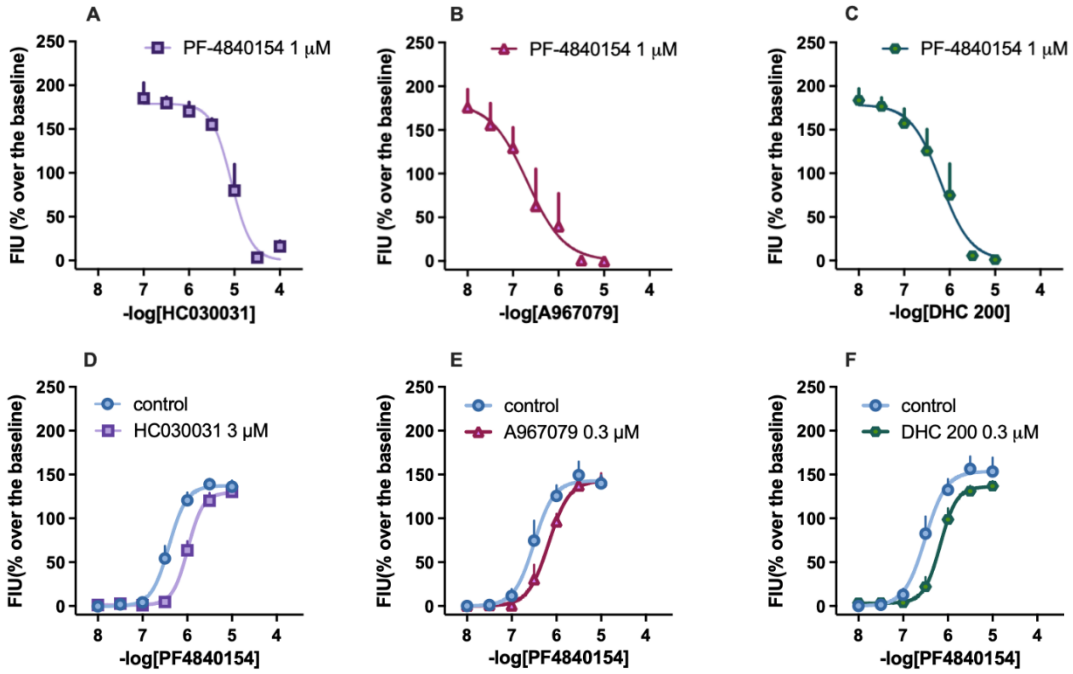

Figure 4. Calcium mobilization assay in A549 cells. The inhibition-response curves experiments are shown in upper panels while the concentration-response curves to PF-4840154 obtained in the absence (control) and presence of HC030031, A967079, and DHC200 are shown in bottom panels. Data are expressed as mean ± sem of at least 4 separate experiments made in duplicate.

Table 2. Potency of standard TRPA1 antagonists.

	pA_2 ($CL_{95\%}$)	pK_B ($CL_{95\%}$)
HC030031	5.65 (5.33 – 5.97)	5.13 (4.20 – 6.05)
A967079	6.29 (5.73 – 6.85)	6.65 (5.28 – 8.03)
DHC200	6.32 (6.15 – 6.49)	6.31 (5.21 – 7.41)

New DHC200 analogs – have been synthesized two series of analogs with two different kinds of modifications to the chemical structure of DHC200. It is not possible, within this work, to show the structures of DHC200-like compounds as they are secreted. The pharmacological activity of the first series of new TRPA1 antagonists is summarized in table 3, while the second series are summarized in table 4.

Table 3. Potency of first series of DHC200 analogs

Compounds	*pA_2 ($CL_{95\%}$)*
DHC200	6.93 (6.61-7.25)
DHC215	6.36 (6.06-6.65)
DHC247	6.37 (5.97-6.95)
DHC248	5.96 (4.42-7.49)
DHC237	6.33 (5.92-6.74)
DHC238	6.40 (5.73-7.07)
DHC243	6.43 (5.94-6.92)
DHC221	5.56 (4.63-6.50)
DHC214	6.12 (5.90-6.34)
DHC250	< 5

Table 4. Potency of second series of DHC200 analogs

Compounds	*pA_2 ($CL_{95\%}$)*
DHC200	6.93 (6.61-7.25)
DHC236	7.34 (6.96-7.72)
DHC252	< 5
DHC253	< 5
DHC254	< 5
DHC255	5.51 (3.85-7.17)
DHC258	6.75 (3.32-10.18)
DHC270	< 5
DHC271	< 5
DHC272	< 5
DHC273	5.31 (4.92-5.70)
DHC274	< 5
DHC275	< 5
DHC276	< 5
DHC277	7.28 (6.88-7.68)
DHC278	< 5
DHC279	< 5
DHC280	< 5

4. Discussions and conclusions

In this work, the pharmacology of the TRPA1 channel receptor was studied through the calcium mobilization assay. This test, performed both through automatize fluorimeters and imaging techniques, was widely used in literature to study TRPA1 agonists (Benemei et al. 2017; Ryckmans et al. 2011) and antagonists (Eid et al. 2008; McNamara et al. 2007; Nassini et al. 2015; Tonello et al. 2017). The cells used to perform the experiments were the A549 (human lung cancer cells) cell line, which natively expresses the receptor (Mukhopadhyay et al. 2011). The expression of TRPA1 in these cells was observed both via western blot experiments (evaluation of protein levels) and real-time PCR (evaluation of mRNA levels). Moreover, Mukhopadhyay and colleagues (2011) have demonstrated TRPA1 receptor functionality in A549 cells using the calcium mobilization assay.

Collectively, in our assay, the natural isothiocyanates AITC and BITC, CA, and the synthetic agonist PF-4840154 behaved as a full agonist of the TRPA1 whit the following rank order of potency: PF-4840154 > BITC ≈ AITC = CA, which is in line with literature data that showed similar potency values. Of note, in our study PF-4840154 showed lower values of pEC_{50} (6.5) than those reported in the literature (7.64) (Ryckmans et al. 2011). This difference can be explained considering that Ryckmans et al. characterized the compound in HEK293 cells transfected with the human isoform of the TRPA1 receptor, while we studied the receptor natively expressed in A549 cells. As far is concerned AITC, the potency obtained in this study is similar to that reported by Mukhopadhyay et al. for the same cells (Mukhopadhyay et al. 2011). In HEK293 cells expressing the recombinant TRPA1 receptor, AITC showed higher potency values, between 5.5 and 6.5 (data summarized in (Baraldi et al. 2010)). When tested on the recombinant receptor, BITC (6.5) showed similar potency to AITC, our data then confirm the literature data that report AITC and BITC as equipotent TRPA1 agonists (Baraldi et al. 2010). CA was also studied in the literature on HEK293 cells expressing the recombinant TRPA1 receptor and showed potency values of about 4.5, therefore in line with our study (Eid et al. 2008; McClenaghan et al. 2012). Our results confirm the potency and full agonist activity of these molecules as TRPA1 agonists. Based on this data, PF-4840154 was selected as the standard agonist for subsequent antagonism experiments.

The standard antagonists that have been studied in this work are A967079, HC030031, and DHC200. All these compounds have been tested using two antagonism protocols: the inhibition protocol, and the Schild protocol. From the inhibition experiments, pK_B was extrapolated equal to 5.13, 6.65, and 6.31 respectively for HC030031, A967079, and DHC200. Regarding the type of antagonism, Schild protocol experiments have shown that

all tested antagonists produced a rightward shift in PF-4840154 concentration-response curve without modifying the agonist maximal effects, demonstrating that their behavior is of a competitive nature. The pA_2 values determined by this protocol are 5.65, 6.29, and 6.32 for HC030031, A967079, and DHC200, respectively. The potency of the antagonists obtained through these two different protocols is very similar to each other. The rank order of potency of the TRPA1 antagonists obtained with these experiments is: DHC200 $\geq$ A967079 > HC030031. In the literature A967079, HC030031 and DHC200 were characterized in HEK293 or CHO cells expressing the human isoform of recombinant TRPA1. Some research groups used different agonists and experimental conditions to study various antagonists. In particular, in the patent reading [i.e. DHC200, WO2010141805] the details of the experimental conditions were omitted. The potency value of antagonists in these publications and/or patents is always expressed as pIC_{50}. To overcome this problem, for each publication and/or patent, the pK_B value has been extrapolated assuming a slope of the concentration-response curve of the agonist of 1. When the potency and concentration values of the agonist used were not explained, we used the potency values reported in the literature and concentration equal to pEC_{80}. The pK_B values thus extrapolated are ~ 8.5 (Chen et al., 2011), ~ 8.5 (WO2010141805) and ~ 6 (McNamara et al. 2007; Nyman et al. 2013) for A967079, DHC200 and HC030031. The rank order of potency obtained according to these data is, therefore: DHC200 = A967079 > HC030031. It is possible to state that the order of potency of antagonists determined in this study, using two different protocols and cells natively expressing the TRPA1 receptor, is in line with data reported in the literature. Overall, the use of standard TRPA1 agonists and antagonists allowed us to demonstrate that our test conditions are suitable for in vitro pharmacological characterization of new TRPA1 ligands in A549 cells.

Intracellular calcium mobilization test, screening of new TRPA1 ligands – Following the set-up of the experimental conditions, new analogous antagonists of DHC200, synthesized by Dr. Delia Preti of the Department of Chemical, Pharmaceutical and Agricultural Sciences of the University of Ferrara, were tested *in vitro* with the intracellular calcium mobilization assay. New compounds, at fixed concentrations, were evaluated against the concentration-response curves of PF-4840154. It is not possible, within this discussion, to show the structures of DHC200-like compounds as they are secreted. The structure of the standard compound has been modified to have two structures with two different heterocycles as can be seen in the figures in table 3 and 4. Of this series of compounds, two, in particular, showed higher potency than the original compound. They are the compounds DHC236 and DHC277 showed a potency value about 3 times higher than DHC200.

In literature, the quest for more selective TRPA1 channel antagonists has led to the development of molecules with optimization of existing scaffolding. Hydra Biosciences was the first company to disclose xanthin derivatives as antagonists to TRPA1, for which HC-030031 is a representative compound. In another study, carried out in our laboratories as part of another project, HC030031 analogous were tested with the calcium mobilization assay. In order to improve the solubility of the compounds, major changes have been made in the imidazole ring, but none of them demonstrated to be active (data not showed). Another xanthin derivative, Chembridge-5861528, was investigated by Orion Pharma as a tool compound. It was shown to be about 10-fold more potent than HC-030031. Another antagonist is the oxime derivative, A-967079 (WO/2009/ 089082) (Chen et al. 2011). In a second series of studies, carried out in our laboratories as part of another project, A967079 analogous, with a change in the oxime (unstable due to the effect of syn-anti isomerism), replaced with a ketone, were tested but none of those compounds showed any pharmacological activity (data not showed). Among the various antagonists that have shown selectively towards TRPA1, only a few have been tested in clinical trials for the treatment of pain or other conditions, but the development of all of them was discontinued because of low solubility or complex pharmacokinetics. A summary of clinical studies for TRPA1 antagonists is reported in (Souza Monteiro de Araujo et al. 2020).

Given that the pronociceptive pathway, involved in various pathophysiological pain conditions, converges in TRPA1 activation, and since all clinical studies concerning new TRPA1 receptor antagonist molecules have been discontinued, new potent and selective compounds, with good pharmacokinetics *in vivo*, are of great interest.

In conclusion, this study allowed the successful development of experimental conditions to pharmacologically characterize new TRPA1 antagonists *in vitro*. As part of this thesis work, they were identified as derivatives of DHC200, 3 times more potent *in vitro* than the original compound. These compounds will be considered a starting point for future studies, and they will be investigated also in vivo migraine models developed in our laboratory. Given the involvement of TRPA1 in several painful (i.e. inflammation, neuropathic pain, migraine) and respiratory disorders (i.e. cough and asthma) compounds of this type could be of great interest as innovative drugs.

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2.4 Preclinical effects of cannabidiol in an experimental model of migraine

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Preclinical effects of cannabidiol in an experimental model of migraine.

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Abstract

Migraine is a disabling disorder characterized by recurrent headaches, accompanied by abnormal sensory sensitivity and anxiety. Despite extensive historical use of cannabis in headache disorders, there is limited research on the non-psychoactive cannabidiol (CBD) for migraine and there is no scientific evidence to prove that CBD is an effective treatment. The effects of CBD are examined here using a calcitonin-gene related peptide (CGRP)-induced migraine model that provides measures of cephalic allodynia, spontaneous pain, altered light sensitivity (photophobia) and anxiety-like behavior in C57BL/6J mice. A single administration of CGRP induced facial hypersensitivity in both female and male mice. Repeated CGRP treatment produced progressively decreased levels in basal thresholds of allodynia in females, but not in males. A single CBD administration protected both females and males from periorbital allodynia induced by a single CGRP injection. Repeated CBD administration prevented increased levels of basal allodynia induced by repeated CGRP treatment in female mice and did not lead to responses consistent with migraine headache increased migraine headache as occurs with triptans. CBD, injected after CGRP, reversed CGRP-evoked allodynia. CBD also reduced spontaneous pain traits induced by CGRP administration in female mice. Finally, CBD blocked CGRP-induced anxiety in male mice, but failed in providing protection from CGRP-induced photophobia in females. These results demonstrate the efficacy of CBD in preventing episodic and chronic migraine-like states with reduced risk of causing medication overuse headache. CBD also shows potential as an abortive agent for treating migraine attacks and headache-related conditions such as spontaneous pain and anxiety.

Introduction

Migraine is a complex nervous system disorder characterized by long-lasting (4-72h) throbbing, unilateral headache often accompanied by nausea, vomiting, sensory amplifications such as hypersensitivity to light, sound, smell and cutaneous allodynia, as well as comorbid anxiety and depression [5; 26]. Migraine affects 12% of the people worldwide and is considered a leading cause of disability, significantly impacting work productivity, as well as well-being and quality of life of patients and their families [22; 32]. Frequency of attacks differs widely, with some people experiencing episodic (1-14 days/month) and other chronic migraine attacks (>15 days with headache/month) [22]. The disorder is highly sex-dependent, with roughly 70% of migraineurs being women [6; 45].

The pathophysiology of migraine is complex and only partially understood. Several lines of evidence suggest that activation of trigeminal nociceptors, which causes a release of vasodilatory neuropeptides including calcitonin gene-related peptide (CGRP) onto the dura and centrally onto the brainstem, plays an important role in the initiation of migraine pain [30; 43]. CGRP is with no doubt central to migraine pathophysiology [50]: first, CGRP levels are increased during a migraine attack [25], returning to normal levels when the attack is resolved [33]. Second, intravenous CGRP infusions can induce migraine-like attacks in individuals who have migraine [37]. Third, CGRP induces cephalic allodynia and light aversive behavior in animal models [19; 40]; and last, CGRP is a relevant target for migraine. Although CGRP receptor antagonists and monoclonal antibodies against CGRP or its receptor offer new options for migraine therapy with improved target specificity [23; 52], there is a great need for new therapeutics.

Early reports indicate that *Cannabis sativa* was used quite extensively as an effective prophylactic and abortive treatment for migraine and other headache disorders [39]. However, the schedule 1 classification of marijuana in 1970 has challenged the clinical evaluation of this substance and its components with respect to headache disorders [39]. We hypothesized here that cannabidiol (CBD), the main non-intoxicating component of cannabis, has anti-migraine effects because of its efficacy for pain-related conditions such as anxiety [18], its analgesic effect associated with the transient receptor potential cation channel subfamily V member 1 (TRPV1) [16], its anti-inflammatory activity shown in an animal model of acute inflammation [15], and its ability to modulate serotonergic transmission [18], which is widely involved in trigeminal activation [28].

We verify our hypothesis by testing CBD efficacy in relieving relevant symptoms of migraine-like states in mice. We first characterize in our laboratory an animal model of acute and chronic migraine that involves measures of periorbital mechanical allodynia associated with systemic administration of exogenous CGRP in female and male mice. We then examine the ability of CBD to modulate CGRP-evoked cephalic allodynia in these predictive acute and chronic migraine models. Finally, we test whether the rescued sensitivity to mechanical stimulation induced by CBD would be accompanied by other anti-migraine CBD actions such as the reversal of CGRP-induced photophobia, spontaneous pain and anxiety-like behavior.

Materials and Methods

Animals. All animals used in the experiments were female and male C57BL/6J mice purchased from Jackson Laboratory (Bar Harbor, ME) or bred in our laboratory. All mice were group housed (3-4/cage) and used for experiments after they reached 9 weeks of age. Animals were kept on a 12-h light/dark cycle with lights off at 7:00 PM in a quiet temperature-controlled room (24°C-25°C). The breeding mice were housed and maintained under identical environmental conditions throughout the study. All animals were naïve to the behavioral procedures except where specified. Experiments were reported in compliance with the ARRIVE guidelines [35]. Group sizes for the main readouts were determined using G Power analysis (3.1 [24]). The total number of mice used throughout the study was N=438. Mice were assigned into the various treatment groups using a random allocation sequence, however ensuring an equal/similar group size (restricted randomization), except where specified. All experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, with methods preapproved by the Institutional Animal Care and Use Committee at Florida Atlantic University.

Drugs. CBD was a generous gift by Sunflora Inc. The CBD powder was obtained by extraction at 97.404% purity (total THC = 0.000%, total cannabinoids = 97.765%). CBD was prepared in a vehicle (VEH) of 3% ethanol, 3% Tween 80, and 0.9% saline, and used at the dose of 10 and 30 mg/kg [12]. CGRP (AnaSpec, Inc) was dissolved in PBS and used at the dose of 0.1 mg/kg [40; 47]. Olcegepant (Tocris) was prepared in 4% DMSO, 4% Tween 80, and 0.9% saline and injected at the dose of 1 mg/kg [19; 20]. Sumatriptan succinate (Fisher Scientific) was dissolved in the same VEH as CBD and used at the dose of 0.6 mg/kg [17]. All compounds were administered in a 5 ml/kg volume injection and given by intraperitoneal (i.p.) route of administration.

Assessment of periorbital mechanical allodynia. The operator who conducted the assessment was always blind to treatment conditions and performed all testing for a given experiment. In the days preceding each experiment, mice were habituated to the testing room twice for 30 min. During this acclimation period mice were gently handled,habituated to i.p. injection and held as during the test, but the filaments were not applied until the test started. On the day of the experiment, 30 additional min of adaptation were given to the mice prior to starting the assessment of mechanical allodynia. In order to decrease the mouse withdrawal reflex, which is usually greater under novelty conditions, two basal points were

conducted; however, only the second baseline was used for data analysis. Female and male mice were tested on separate days by the same operator. Allodynia was evaluated in the mouse periorbital region over the rostral portion of the eye and near the midline. Mice, which during acclimation period were left undisturbed inside Plexiglas cylinders covered on top by a meshed grid, at the time of assessment were placed on top of the grid and gently held by the tail with one hand to keep them from slipping away. With the palm of the same hand, the animal was covered without crushing it, leaving it free of all movements. With the other hand, von Frey filaments of different forces (0.02, 0.04, 0.07, 0.16, 0.4, 1.0, and 1.4 g) were applied to the periorbital area perpendicular to the skin, with sufficient force to cause slight buckling, and held for approximately 2 sec to elicit a positive response. Mice were poked five times with the same filament in a uniform manner throughout the periorbital region before changing filament. The stimulation was initiated with the 0.16 g filament. A response occurred when the mouse stroked the face with its forelimb, withdrew its head from the stimulus or shook its head [19]. Allodynia was measured according to the up-down method [13]. The absence of response led to the use of a filament with increased force, whereas a response led to the use of a lighter filament. After the first response occurred, four more measurements were collected for each mouse or until four consecutive positive or negative responses occurred. The 50% mechanical withdrawal threshold (expressed in g) was then calculated [14]. Periorbital mechanical allodynia was assessed in a total of N=257 mice. Mice were allocated to vehicle or treatment groups by counterbalancing animal performance into the different experimental groups to ensure no threshold difference at baseline.

Grimace Face Assay. Female and male C57BL/6J mice were acclimated for 30 min inside Plexiglas cylinders covered on top by a meshed grid. Mice received a pre-treatment of either CBD (30 mg/kg) or VEH, and, 30 min later, received a treatment of CGRP (0.1 mg/kg) or PBS. To record facial grimace, mice were videotaped for 30 sec at 15 min, 30 min, and 1-h post-injection. The videos were then used to take still images that included the appearance of the mouse's face, ears, and posture. The pictures were randomized, and a blind evaluator scored each picture on a scale of 0-2 based on signs of a facial grimace. The criteria were based on orbital tightening, nose bulge, cheek bulge, ear position, and whisker change [36]. Scores were averaged across each treatment and analyzed to determine facial grimace. A total of N=63 mice were used for this experiment.

Photophobia. Photosensitivity was tested in the dark light box (DLB). Two Med Associates activity monitor chambers with dark box inserts were used. The testing chambers were

placed in ventilated and sound attenuating cubicles (56 × 38 × 36 cm). Each chamber had two compartments equivalent in dimensions and connected to each other with an opening. One compartment was delimited by a black Plexiglas insert to prevent light entrance (dark compartment), while the other (light compartment) was lit using a 1000 lux lighting unit (surgical lamp, Fisher Scientific) [53]. Infrared beams allowed for tracking the animal's movements and time spent in each compartment. The percent of time spent in the light compartment was calculated and served as a measure of photophobia. Following the photophobia test, mice were returned to their home cages.

Anxiety-like behavior. Anxiety-like responses were assessed by the elevated plus maze (EPM) test as we previously described [53]. The EPM apparatus was a black, 'plus'-shaped platform equipped with two open arms and two closed arms of the same dimensions (35.6 x 7.6 cm). The platform was at a raised height of 50 cm above the ground and placed under room lighting of 500 lux. The EPM test was conducted using the same mice (on the same drug treatments) that were previously used for the photophobia test. Following 5 min resting in their home cages, the mice were placed on the central platform of the EPM apparatus faced to a closed arm for 5 min. An operator blinded to the treatment schedule recorded the time spent onto the open arms of the maze as well as the entries onto the open and closed arms. The percent of open arm time (OAT) and entries (OAE) served as measures of anxiety-like activity, whereas the number of closed arm entries (CAE) was an indicator of the mouse locomotor behavior. Anxiety-like behavior and photophobia were tested in the same animals (Total mice: N=95).

Open field test. Locomotor activity chambers from Med Associates (St Albans, Vermont) were used to track and analyze the mouse locomotor behavior. This experiment was performed in N=23 male mice (7-8/treatment) using similar methods as we previously described [7]. On day 1, mice were habituated to the open field chambers for 1h. On day 2, locomotor activity was tested for 30 min 1h after receiving an i.p. injection of CBD (10 mg/kg, 30 mg/kg) or VEH. Total distance traveled (cm) and immobility time (sec) served as primary readouts for the mouse locomotor behavior.

Statistical Analysis. Tibco Statistica (Version 13.5.0.17) was used for data analysis. Non-parametric statistical tests were used to analyze thresholds of mechanical stimulation of the periorbital region and grimace face scores. Specifically, the Friedman test was used to determine overall differences in treatments across multiple measures. This test was followed

up by the Wilkoxon test when significance was detected. The Kruskall-Wallis test was used to determined overall differences in multiple independent groups. Mann-Whitney U test was conducted to examine comparisons between groups and to analyze differences in basal responses between female and male mice. Two-way ANOVA was used for analysis of photophobia in the DLB and anxiety-like behavior in the EPM, followed, when appropriate, by Fisher LSD post-hoc comparisons. Open field data were analyzed by means of a 1-way ANOVA using “treatment” as a between-subject factor. Linear regression analyses were conducted to determine correlations between variables. Significance was set at $p<0.05$.

Results

Effects of acute CGRP treatment on periorbital allodynia in female and male mice. Female and male mice received a single i.p. injection of CGRP or PBS and periorbital mechanical allodynia was assessed (**Figure 1A**). Friedman test conducted in PBS-treated female mice revealed similar mechanical thresholds across the various time points ($X^2(4)=2.222$, $p=0.694$). In contrast, significant changes in allodynic responses following CGRP administration were observed ($X^2(4)=16.915$, $p=0.00201$). Mechanical sensitivity was significantly increased at 0.5h ($p=0.0179$) and 1h ($p=0.0117$), but not at 2h ($p=0.0796$) following CGRP administration. Comparisons between PBS- and CGRP-treated samples led to similar results (basal sensitivity threshold (B2): $p=1.000$; 0.5h: $p=0.0215$; 1h: $p=0.0138$; 2h: $p=0.237$; 24h: $p=1.000$, **Figure 1B**).

Friedman analysis conducted in PBS-treated male mice revealed similar mechanical thresholds across the various time points ($X^2(4)=3.027$, $p=0.553$), whereas significant changes in allodynic responses following CGRP administration were noticed ($X^2(4)=21.327$, $p=0.00027$). Changes in allodynic responses following CGRP administration were observed at 0.5h and 1h ($p=0.00963$ for both time points), as well as at 2h ($p=0.0218$). Comparisons between PBS- and CGRP-treated groups roughly confirmed the results (B2: $p=0.839$; 0.5h: $p=0.0153$; 1h: $p=0.00149$; 2h: $p=0.148$; 24h: $p=0.817$, **Figure 1C**). Subsequent analysis conducted on basal responses of female and male mice indicated that the latter were more sensitive in showing responses to Von Frey hairs than their female counterparts ($U=117.0$, $p=0.00127$).

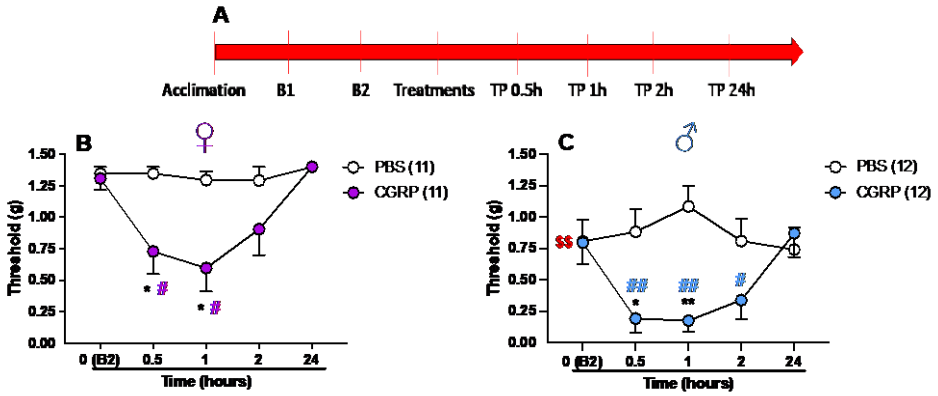

Figure 1. A single intraperitoneal (i.p.) administration of calcitonin gene-related peptide (CGRP) induces mechanical hypersensitivity in the periorbital region of C57BL/6J mice. (**A**) Experimental timeline: Following acclimation, two basal points of periorbital mechanical allodynia were calculated. Female and male mice were then i.p. injected with CGRP (0.1 mg/kg) or VEH (PBS). Assessment of periorbital mechanical allodynia was evaluated again 0.5h, 1h, 2h and 24h after treatments. Von Frey hairs were applied onto the periorbital area of (**B**) female and (**C**) male mice using the up-down method. Sensitivity thresholds (Force, g) were assessed at 30 min (0.5 hours), 1, 2 and 24 hours following acute administration of either 0.1 mg/kg CGRP or PBS (N=11 vs. 11 females and 12 vs.12 males. Total mice: N=46). *p<0.05, **p<0.01 difference from PBS. $^{\#}$p<0.05, $^{\#\#}$p<0.01 difference from basal sensitivity thresholds (B2). $^{\$\$}$p<0.01 difference between sexes. TP, Time point.

Effects of chronic CGRP treatment on periorbital allodynia in female and male mice. CGRP or PBS were administered to female and male mice every day for 4 consecutive days to model the progression from episodic to chronic migraine (**Figure 2A**). PBS-treated female mice were insensitive to the mechanical stimulation throughout the 4 days of treatment, at all the time points examined (0.5h: $X^2(4)= 3.000$, p=0.557; 1h and 24h: $X^2(4)=4.000$, p=0.406). Females treated with CGRP over 4 days were sensitive at 0.5h ($X^2(4)=15.240$, p=0.00423). Mechanical sensitivity reached significance as compared to B2 (p=0.0277) and PBS (p=0.0088) on day 4, **Figure 2B**). At 1-h post-treatment, CGRP produced mechanical allodynia (X^2 (4)=15.636, p=0.00355) on days 1 (p=0.0277 from B2, p=0.0088 from PBS), 3 (p=0.0431 and p=0.0409 from B2 and PBS, respectively) and 4 (p=0.0431 from B2 and p=0.0298 from PBS, **Figure 2C**). Repetitive CGRP administration over 4 days produced progressively increased basal levels of mechanical allodynia at 24-h post-treatment (p=0.0431 on days 2 and 3, and p=0.0277 on day 4 vs. B2; p=0.0298 on day 2, p=0.0552 on day 3, p=0.0088 on day 4 vs. PBS-treated mice, **Figure 2D**).

Male mice were less sensitive than females to the effect of CGRP across the 4 treatment days. Overall, the effect of CGRP was not significant at 0.5h ($X^2(4)=8.521$, p=0.0742, **Figure 2E**), 1h ($X^2(4)=9.118$, p=0.058, **Figure 2F**) and 24h ($X^2(4)=4.626$, p=0.327, **Figure 2G**). The lack of hypersensitivity noticed in the CGRP group at 0.5 and 1h (as well as the

lack of effect at 0.5 and 1h on day 2 seen in females) suggests inconsistent outcomes to CGRP treatment particularly when mice are no longer naïve to the acute effects of CGRP. Surprisingly, changes in mechanical thresholds were observed in PBS-treated males at all time points (0.5h: $X^2(4)=12.833$, $p=0.0121$; 1h: $X^2(4)=11.837$, $p=0.0186$; 24h: $X^2(4)=12.888$, $p=0.0118$) as the effects of mechanical stimulation became less meaningful across days in PBS-treated male mice ($p=0.0431$ vs. B2 on days 2 and 3 at 0.5h, on days 1 and 4 at 1h and 24h). . Further analysis displayed differences between PBS- and CGRP-treated males at the same time points (0.5h: $p=0.0405$ and $p=0.0135$ on days 2 and 3, respectively; 1h: $p=0.0135$ on days 1 and 4; 24h: $p=0.0135$ and $p=0.0239$ on days 1 and 4). Analysis performed on basal responses between female and male mice indicated, once again, that males were spontaneously more sensitive than females ($U=42.0$, $p=0.00386$).

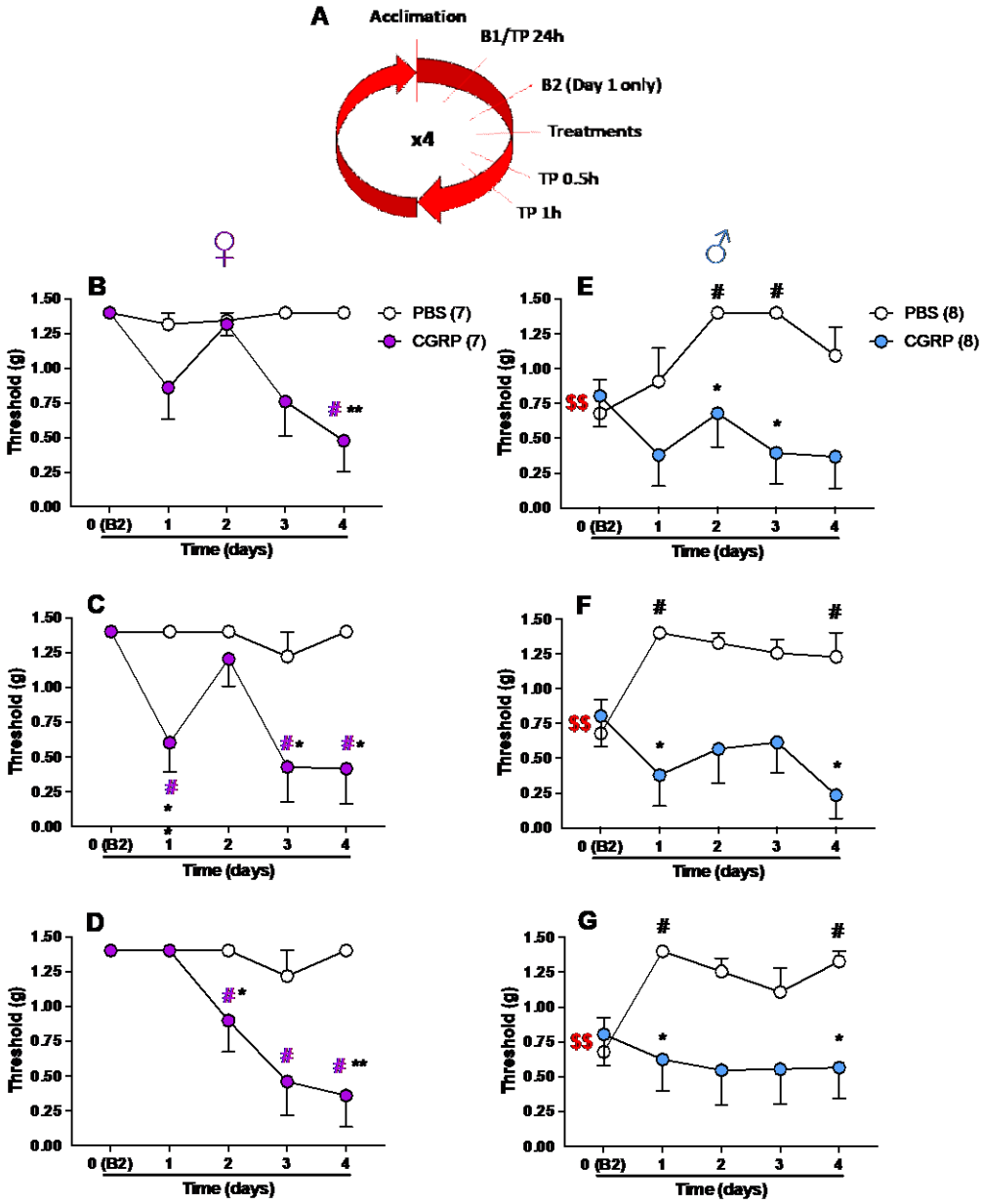

Figure 2. Repeated CGRP treatment produces progressively decreased levels of basal thresholds of allodynia in females, but not male C57BL/6J mice. **(A)** Experimental timeline: To model the progression from episodic to chronic migraine, CGRP (0.1 mg/kg) or PBS were administered to female and male mice every day for 4 consecutive days (x4). On day 1, following acclimation, mechanical thresholds were evaluated twice prior to 0.5h, 1h and 24h after treatments. On days 2, 3 and 4, an identical procedure was used except that only one basal score was recorded prior to treatment. The basal score recorded from day 2 matched the 24h time point for each day. **(B&E)** Thresholds of mechanical allodynia assessed at 0.5h in female and male mice, respectively. **(C&F)** Thresholds of mechanical allodynia assessed at 1-h post-treatment time in female and male mice, respectively. **(D&G)** Thresholds of mechanical allodynia assessed at 24-h post-injections in female and male mice, respectively. Results are the mean ± SEM of Force (g) applied through von Frey filaments using the up-down method to N=14 female (7/group) and 16 male (8/group) mice (Total mice: N=30). *p<0.05, **p<0.01 difference from PBS. $^{\#}$p<0.05 difference from basal threshold (B2). $^{\$\$}$p<0.01 difference between sexes. TP, Time point.

Effect of CBD on locomotor activity. Open field test was carried out to determine whether CBD at doses of 10 and 30 mg/kg affected animal locomotor activity. Mean total distance traveled (cm) ± SEM was: 3269.3 ± 246.7 (VEH), 3207.1 ± 366.1 (CBD 10) and 3106.3 ± 459.7 (CBD 30). Mean immobility time (sec) ± SEM was: 1210.6 ± 27.3 (VEH), 1223.9 ± 47.5 (CBD 10) and 1243.7 ± 36.9 (CBD 30). CBD did not significantly alter total distance traveled [$F(2,20)=0.0506$; $p=0.950$] or time of immobility [$F(2,20)=0.183$; $p=0.834$] in the open field assay.

Effect of CBD on CGRP-induced periorbital allodynia in female and male mice. Experimental timeline is shown in **Figure 3A**. Friedman test conducted in CGRP-treated female mice revealed significant increase in mechanical sensitivity as compared to the basal threshold ($X^2(3)=9.842$, $p=0.0199$) at 1h ($p=0.0346$) and 2h ($p=0.0464$) from the single CGRP administration. Analysis performed between all treatment groups confirmed there was difference in the mechanical thresholds at 1h ($H(3)=17.790$, $p=0.0005$) and 2h ($H(3)=8.093$, $p=0.0441$). Single between-group comparisons indicated that administration of CGRP increased sensitivity to von Frey stimulation as compared to PBS control at 1h ($p=0.0192$), but not at 2h ($p=0.0773$). Both doses of CBD prevented the increased sensitivity evoked by the administration of CGRP at 1h (CBD 10: $p=0.0192$; CBD 30: $p=0.030$, **Figure 3B**).

Overall, the analysis performed in male mice between all treatment groups revealed significant changes in allodynic responses at 0.5h ($H(3,)=14.319$, $p=0.0025$), 1h ($H(3,)=12.065$, $p=0.0072$) and 2h ($H(3)=20.092$, $p=0.0002$) from CGRP injection. Consistently, between group comparisons indicated decreased thresholds for CGRP-treated mice as compared to PBS-treated mice at all time points examined (0.5h: $p=0.00406$, 1h: $p=0.00541$ and 2h: $p=0.00276$). This effect was prevented by both CBD doses at all time points (CBD 10: $p=0.00493$ (0.5h), $p=0.030$ (1h), $p=0.00304$ (2h); CBD 30: $p=0.0109$ (0.5h), $p=0.0275$ (1h), $p=0.00493$ (2h), **Figure 3C**). Further analysis of basal responses conducted between sexes confirmed significant difference in spontaneous nociceptive response to mechanical stimulation of the mouse periorbital region ($U=308.0$, $p=0.00003$).

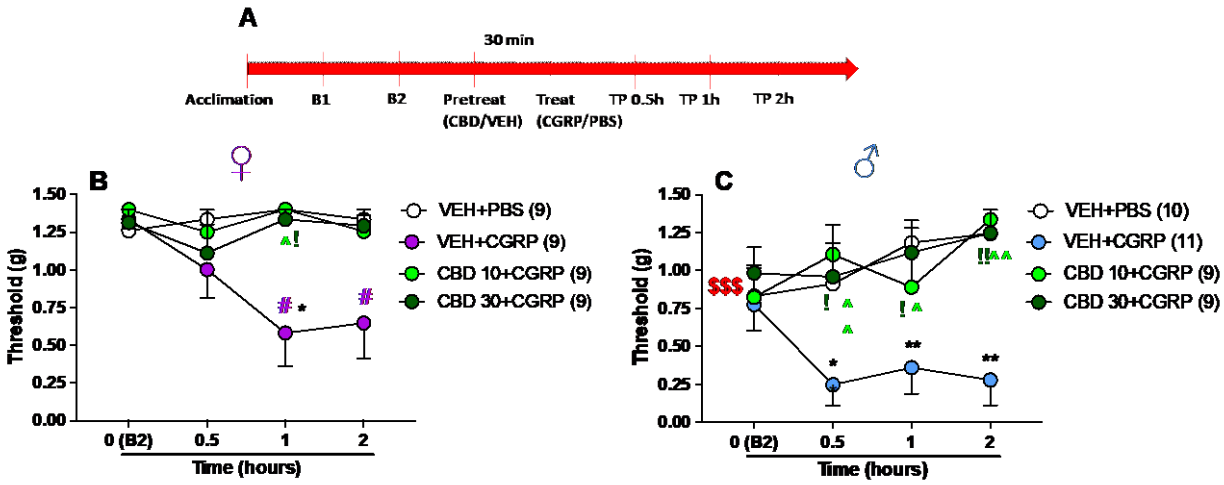

Figure 3. Cannabidiol (CBD) prevents mechanical hypersensitivity induced by a single i.p. CGRP injection in C57BL/6J mice. (A) Experimental timeline: Following determination of two basal threshold measures, female and male mice were i.p. administered with CBD (10 mg/kg or 30 mg/kg) or VEH of CBD, 30 min prior to i.p. administration of CGRP (0.1 mg/kg) or PBS. Periorbital mechanical allodynia was evaluated 0.5h, 1h and 2h after the last drug administration. Results are the mean ± SEM of Force (g) applied using the up-down method to **(B)** N=36 female (9/group) and **(C)** 39 male (9-11/group) mice (Total mice: N=75). *p<0.05, **p<0.01 difference from PBS. #p<0.05 difference from basal thresholds (B2). ^,!p<0.05 ^^,!!p<0.01 difference form CGRP. $$$p<0.001 difference between sexes. TP, Time point.

Effect of repeated CBD administration on increased levels of basal periorbital allodynia induced by repeated CGRP treatment in female mice. Experimental timeline is shown in **Figure 4A**. At 1-h post-injection, the CGRP-treated group showed increased sensitivity to mechanical stimulation ($X^2(4)=10.851$, p=0.0282) across all treatment days (day 1: p=0.0277; days 2, 3 and 4: p=0.0425). Multiple independent group analysis was significant for day 1 (H(3)=7.931, p=0.0475), day 2 (H(3)=11.041, p=0.0115), day 3 (H(3)=10.754, p=0.0131) and day 4 (H(3)=13.047, p=0.0045). Between-group comparisons indicated a significant difference between the groups receiving PBS and CGRP on treatment day 3 (p=0.0239), which was blocked by both CBD doses (CBD 10: p=0.0239; CBD 30: p=0.0313, **Figure 4B**).

At 24-h post-injection, when the acute effects of the treatments wear off, repetitive CGRP administration over 4 days produced a significant decrease in basal thresholds of mechanical allodynia ($X^2(4)=14.305$, p=0.00638) with progressively increased levels of allodynia that became significant on day 4 (p=0.0250). Multiple independent group analysis was significant for days 3 (H(3)=7.954, p=0.0470) and 4 (H (3)=19.883, p=0.00002) with group comparisons indicating remarkable effect of repeatedly injected CGRP on day 4

(p=0.00457), which was prevented by pretreatment of both CBD 10 (p=0.00387) and 30 mg/kg (p=0.00538), **Figure 4C**.

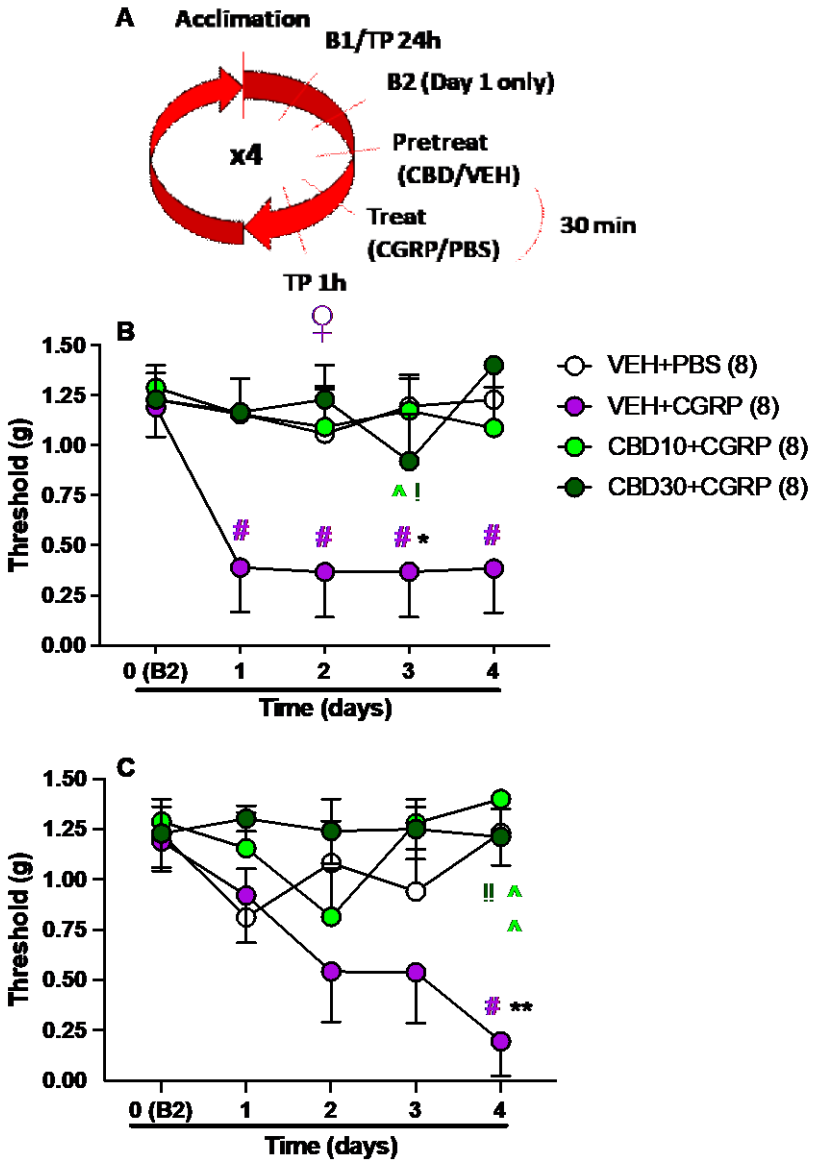

Figure 4. Repeated CBD administration prevents increased levels of basal allodynia induced by repeated CGRP treatment in females C57BL/6J mice. **(A)** Experimental timeline: Female mice were i.p. administered daily with CBD (10 mg/kg, 30 mg/kg) or VEH and with CGRP (0.1 mg/kg) or PBS 30 min later for 4 consecutive days (x4). On day 1, two basal measures of facial allodynia were determined immediately prior to the treatments as well as at 1-h and 24-h post-treatments. On days 2, 3 and 4, the same procedure was repeated except that only one basal measure of allodynia was taken, matching the 24h post-treatment time point. Thresholds of facial allodynia were assessed **(B)** 1h and **(C)** 24h after the injections. Results are the mean ± SEM of Force (g) applied through von Frey filaments using the up-down method to N=32 (8/group) female mice. *p<0.05, **p<0.01 difference from PBS. #p<0.05 difference from basal thresholds (B2). ^,!p<0.05 ^^,!!p<0.01 difference form CGRP. TP, Time point.

Effect of sustained CBD administration in female mice not receiving CGRP.

Female mice not treated with CGRP received repeated injections of CBD, sumatriptan or VEH (**Figure 5A**) and periorbital mechanical allodynia was examined. At 1-h post-injection, repeated treatment with CBD did not alter periorbital withdrawal thresholds ($X^2(6)=6.000$, $p=0.423$) across days, whereas repeated sumatriptan injections produced significant reductions of the mechanical thresholds ($X^2(6)=22.787$, $p=0.00087$) on days 3 ($p=0.0277$), 4 and 8 ($p=0.0431$), and 5 ($p=0.0179$), as compared to B2. On days 3 ($p=0.0238$) and 5 ($p=0.00813$), mechanical thresholds were significantly decreased as compared to VEH (**Figure 5B**).

Evaluation at 24h after injections led to similar results with repeated CBD treatment that did not alter sensory thresholds ($X^2(6)=8.366$, $p=0.212$) while sumatriptan decreased mechanical thresholds ($X^2(6)=23.793$, $p=0.00057$) on days 4 ($p=0.0179$), 7 and 8 ($p=0.0277$ both days) as compared to B2. On day 4, mechanical thresholds were significantly decreased as compared to VEH ($p=0.00813$, **Figure 5C**). Thresholds remained lower for several days following the termination of sumatriptan (day 10 and 16: $p=0.0179$ vs. B2 and $p=0.00813$ vs. VEH; day 22: $p=0.108$ vs. B2 and $p=0.272$ vs. VEH).

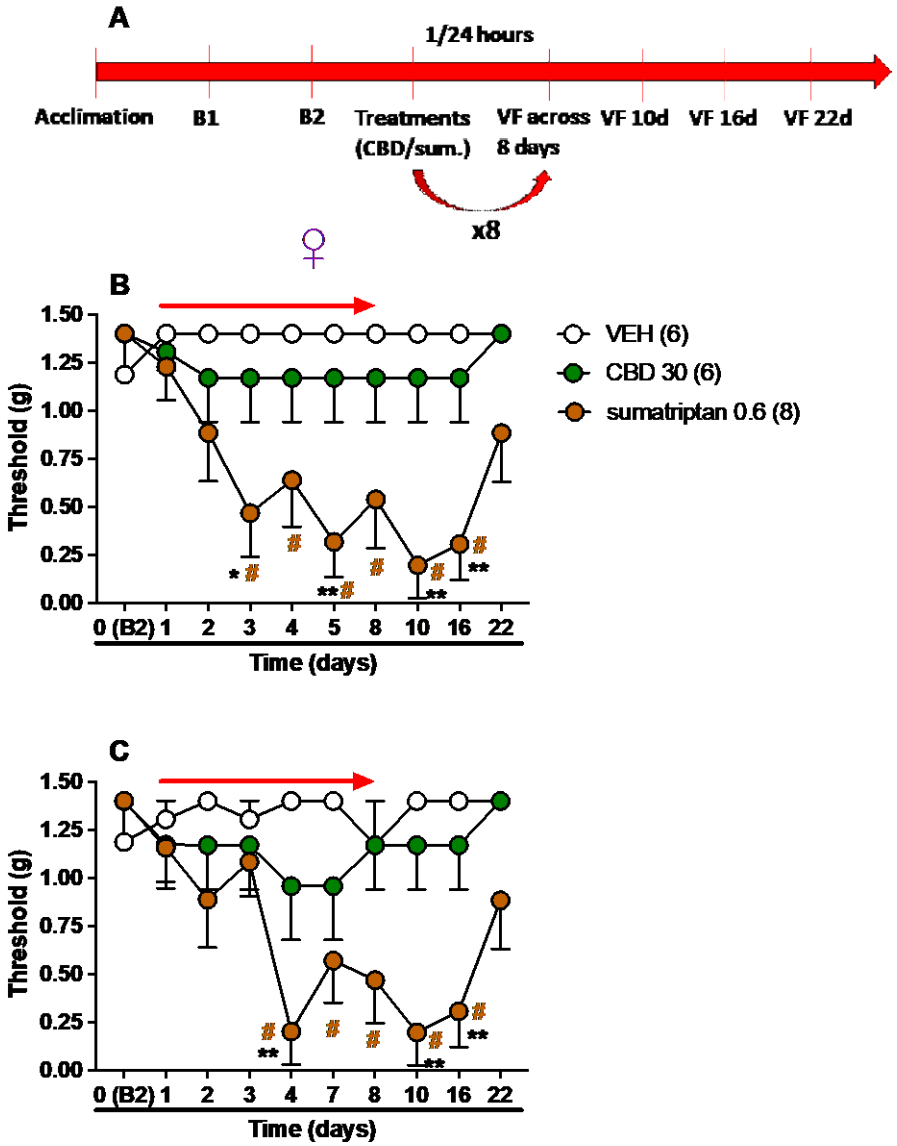

Figure 5. As opposed to sumatriptan, 8-day exposure to CBD does not alter sensory thresholds to mechanical stimulation of the periorbital region. **(A)** Experimental timeline: Female mice were i.p. administered daily for 8 days (x8) with CBD (30 mg/kg), sumatriptan (0.6 mg/kg) or VEH, and withdrawal thresholds were measured 1h and 24h after the injections. Periorbital withdrawal thresholds were evaluated again after the treatment period on days 10, 16 and 22. **(B)** Assessment of periorbital mechanical allodynia at 1-h post-injection. **(C)** Assessment of allodynia 24-h post-injection. Results are the mean ± SEM of Force (g) applied through von Frey filaments using the up-down method to N=20 mice (6-8/group). *p<0.05, **p<0.01, difference from VEH; #p<0.05 difference from basal thresholds (B2). Red arrow, 8-day treatment period; VF, von Frey.

Effect of CBD as an abortive treatment for CGRP-evoked periorbital allodynia in female mice. In this experiment, mice received CBD after CGRP (**Figure 6A**). CGRP-treated female mice showed a significant increase in mechanical sensitivity as compared to the basal threshold ($X^2(2)=10.800$, p=0.00452), as assessed 0.5h and 1h (p=0.0277 both times) following VEH injection. Multiple group analysis indicated differences in allodynic responses at 0.5h (H(3)=9.924, p=0.0192) and 1h (H(3)=14.423, p=0.0024). As shown in **Figure 6B**, the CGRP+VEH group displayed increased mechanical sensitivity as compared to the PBS+VEH group at both 0.5h and 1h time points (p=0.0208). CBD, administered 30 min after CGRP, successfully blocked the CGRP-evoked allodynia at both doses of 10 (1h: p=0.0356) and 30 mg/kg (0.5h: p=0.0356; 1h: p=0.0135).

In a similar experiment conducted with the CGRP receptor antagonist olcegepant (experimental timeline in **Figure 6C**), female mice treated with CGRP displayed a significant increase in mechanical sensitivity as compared to the basal threshold (overall, $X^2(3)=18.6$, p=0.00033; 0.5h: p=0.0277; 1h: p=0.0179; 2h: p=0.0117). Multiple group analysis confirmed differences in allodynic responses at 0.5h (H(2)=6.253, p=0.0439), 1h (H(2)=11.946, p=0.0025) and 2h (H(2)=15.837, p=0.0004). CGRP was effective across all time points (0.5h: p=0.045; 1h: p=0.000982; 2h: p=0.00241). Olcegepant successfully blocked allodynia at 1-h (p=0.00865) and 2-h (p=0.00136) post-injection (**Figure 6D**).

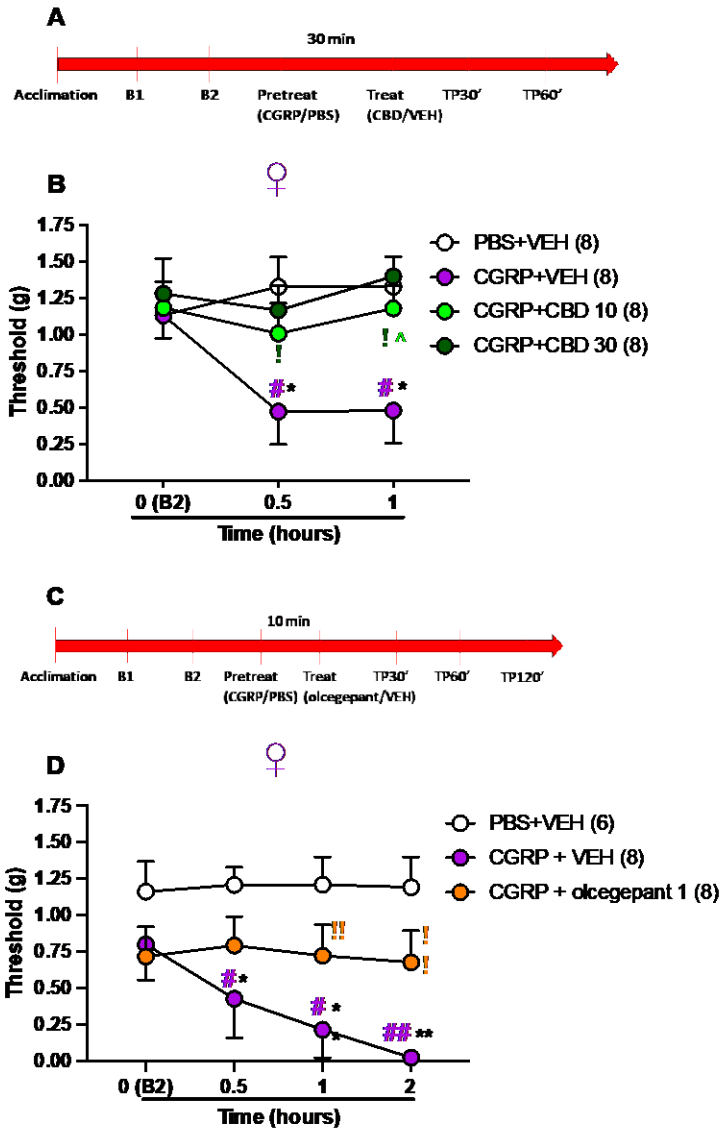

Figure 6. CBD can serve as an abortive treatment for CGRP-evoked hypersensitivity in female C57BL/6J mice. **(A&C)** Experimental timeline: Basal threshold of responses to mechanical stimulation were recorded in all mice. A single injection of CGRP (0.1 mg/kg) or PBS was then conducted 30 min prior to administration of CBD (10 mg/kg and 30 mg/kg) or VEH. Mechanical allodynia was assessed again at 0.5h and 1h from the last injection, that is 1h and 1.5h from the CGRP or PBS injection. In a separate experiment olcegepant (1 mg/kg) was injected 10 min after PBS or CGRP (0.1 mg/kg), with cephalic allodynia assessed 0.5h, 1h and 2h later. **(B&D)** Both CBD and olcegepant, successfully blocked the CGRP-evoked allodynia. Results are the mean ± SEM of Force (g) applied through von Frey filaments using the up-down method to female mice (Total: N=54, 32 for CBD and 22 for olcegepant testing; 6-8/group). *p<0.05, **p<0.01 difference from PBS. #p<0.05, ##p<0.01 difference from basal thresholds (B2). ^,!p<0.05, !!p<0.01 difference form CGRP. TP, Time point.

Effect of CBD on CGRP-induced facial grimace in female and male mice. Experimental timeline is shown in **Figure 7A**. Overall changes in grimace scores were observed in female mice 15 and 60 min following treatments (H(3)=21.833, p=0.0001 and H(3)=15.581, p=0.0014, respectively). Specifically, clear signs of facial discomfort were induced by peripherally-administered CGRP at 15 min (p=0.00457) and 60 min (p=0.00644). These signs were attenuated at 60 min by systemic administration of CBD (p=0.0405, **Figure 7B**), suggesting that CBD contributes to reduce spontaneous pain, a response consistent with migraine. In male mice, differences in grimace scores were evident only at the 15 min time point (H(3)=10.044, p=0.0182) due to CGRP treatment (p=0.0206). Signs of facial pain were not rescued by CBD (p=0.772, **Figure 7C**).

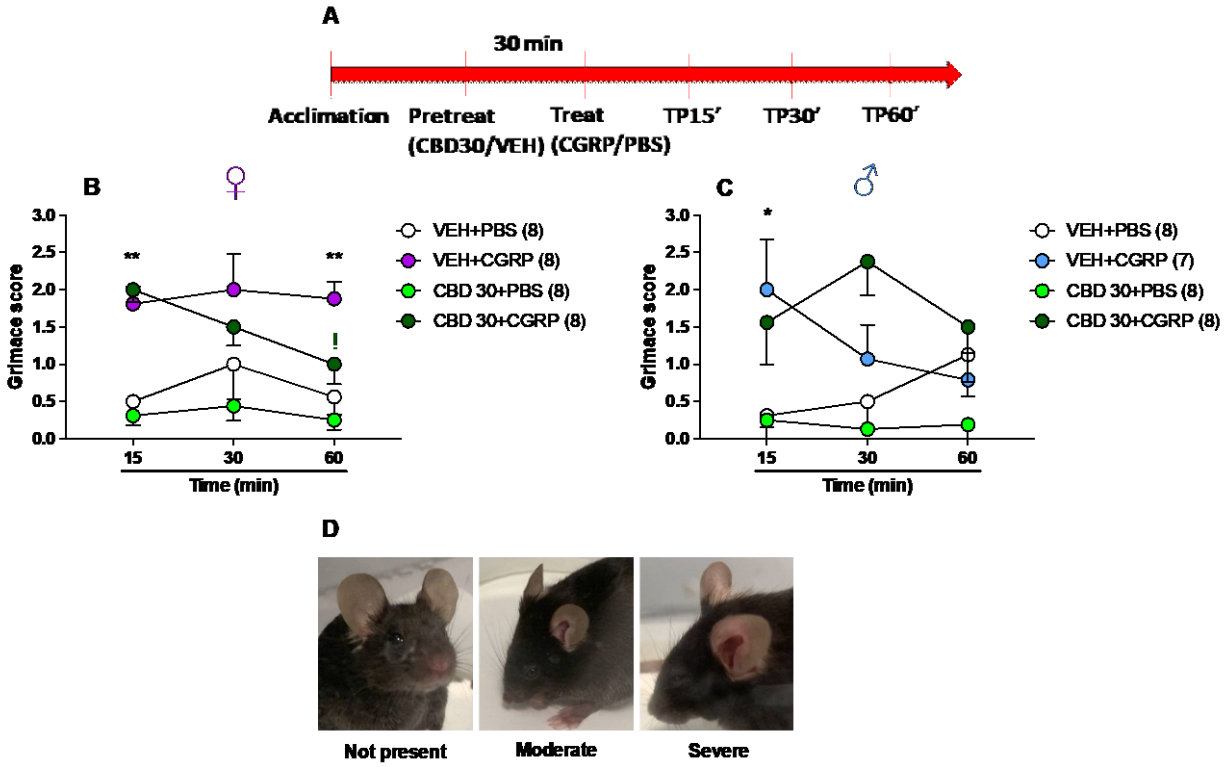

Figure 7. CBD reduces spontaneous pain traits induced by CGRP administration in female C57BL/6J mice. **(A)** Experimental timeline: Mice received an i.p. injection of CBD (30 mg/kg) or VEH prior to i.p. administration of CGRP (0.1 mg/kg) or PBS. Five facial features such as orbital tightening, nose bulge, cheek bulge, ear position and whisker change were scored 0-2 (0: not present; 1: moderately visible; 2: severe) by two blinded individuals 15, 30 and 60 min after the last injection. Values are the mean ± SEM of the grimace scores (previous score averages of individual readers were averaged for an overall score at each time point) observed in **(B)** female (N=32, 8/group) and **(C)** male (N=31, 7-8 group) mice (Total: N=63). (**D**) Three-point scale (not present - 0; moderate - 1; severe - 2) used to calculate the grimace scores. *p<0.05, **p<0.01 difference from VEH+PBS. !p<0.05 difference form CGRP. TP, Time point.

Effect of CBD on CGRP-induced photophobia in male and female mice using DLB.

Experimental timeline is shown in **Figure 8A**. ANOVA revealed a main "treatment" (CGRP vs. PBS) effect [$F(1,44)=6.654$, $p=0.0133$] accompanied by lack of "pretreatment" (CBD vs. VEH) effect [$F(1,44)=0.653$, $p=0.423$] or interaction [$F(1,44)=0.177$, $p=0.675$] in female mice, suggesting that CGRP led the animals to spend significantly less time in the light compartment of the DLB and that CBD failed to reverse the aversion to light induced by CGRP (**Figure 8B**). The same analysis conducted in male mice led to a lack of significant "treatment" effect [$F(1,43)=3.421$, $p=0.0712$], accompanied by not significant "pretreatment" effect [$F(1,43)=0.224$, $p=0.638$] or interaction [$F(1,43)=1.090$, $p=0.302$] (**Figure 8C**).

Effect of CBD on CGRP-induced anxiety in male and female mice using EPM.

ANOVA conducted on the EPM-related variables revealed no changes in % OAT in female mice ("pretreatment" [$F(1,44)=0.749$, $p=0.391$], "treatment" [$F(1,44)=0.687$, $p=0.411$], interaction [$F(1,44)=0.005$, $p=0.941$], **Figure 8D**). In contrast, the same analysis conducted in male mice revealed changes in % OAT ("pretreatment x treatment" interaction [$F(1,43)=4.430$, $p=0.0411$], **Figure 8E**). Likewise, analysis of % OAE led to no changes in females ("pretreatment" [$F(1,44)=0.117$, $p=0.733$], "treatment" [$F(1,44)=0.240$, $p=0.626$], interaction [$F(1,44)=0.701$, $p=0.406$], **Figure 8F**), and rescued CGRP-induced reduction in % OAE in male mice ("pretreatment x treatment" interaction [$F(1,43)=5.225$, $p=0.0272$], **Figure 8G**). On post hoc analysis, CGRP led to anxiogenic-like activity ($p<0.05$ for both % OAT and OAE), which was blocked by CBD pretreatment ($p<0.05$ for both variables). Analysis of CAE, an indicator of the mouse locomotor behavior, led to similar results in females and males, with CGRP eliciting a reduction in the CAE as compared to PBS-treated mice (females: [$F(1,44)=8.646$, $p=0.00520$], males: [$F(1,43)=7.018$, $p=0.011$], **Figures 8H&I**).

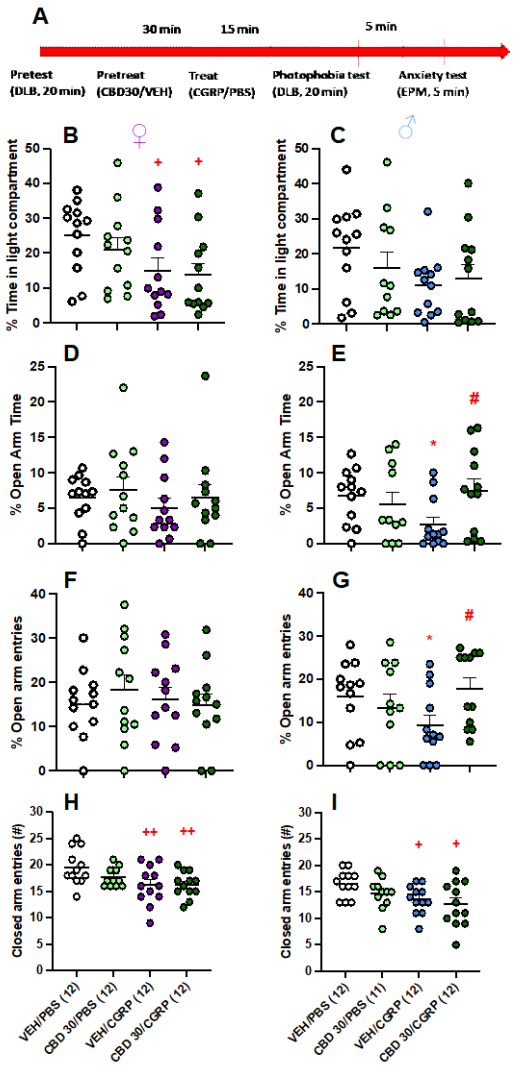

Figure 8. CBD fails to block CGRP-induced photophobia in female, while successfully reverses CGRP-induced anxiety-like behavior in male C57BL/6J mice. **(A)** Experimental timeline: Mice (Total: N=95) of both sexes (females: N=12/group; males: N=11-12/group) were allowed to explore the dark light box (DLB) in a 20-min pretest session. The day after, the mice received i.p. pretreatment of CBD (30 mg/kg) or VEH and, 30 min later, i.p. treatment of CGRP (0.1 mg/kg) or PBS. Fifteen min after the last injection, mice were allowed to re-experience the DLB for 20 min. Following the photophobia test, mice were returned to their home cages. Five min later, a 5-min elevated plus maze (EPM) was performed in the same animals. **(B)** CGRP (0.1 mg/kg) induced significant photophobia that was not prevented by CBD pretreatment in female mice. **(C)** The same CGRP dose did not produce a significant light aversive behavior in male mice. **(D)** CGRP failed to produce anxiogenic-like effect in female mice as shown by the percent (%) of time spent exploring the open arms of the EPM (open arm time). However, **(E)** CGRP led to reduced % open arm time, rescued by CBD pretreatment, in male mice. **(F)** CGRP failed to produce changes in the % open arm entries in females, whereas **(G)** induced reduced % open arm entries in males, an effect reversed by CBD pretreatment. **(H)** CGRP-treated females showed decreased exploration of the EPM maze (decreased closed arm entries). **(I)** The elevated anxiety of CGRP-treated male mice was accompanied by a decreased number of closed arm entries. Values are presented as mean percent (%) of time spent in the light compartment (DLB) and % ± SEM of open arm time and entries and mean ± SEM number of closed arm entries (EPM). +$p<0.05$, ++$p<0.01$ difference from PBS-treated groups; *$p<0.05$ vs. VEH/PBS; #$p<0.05$ difference VEH/CGRP-CBD 30/CGRP.

Anxiety-photophobia correlations. Linear regression analyses were conducted in VEH/PBS-treated groups to determine correlations between photophobia- and anxiety-related variables. As shown in **Figure 9**, in neither female or male mice there was significant correlation between the % of time spent in the light compartment of the DLB and the % OAT on the EPM (**A**, females: R^2=0.00580, p=0.814; **B**, males: R^2=0.0471, p=0.497), or % OAE (**C**, females: R^2=0.0147, p=0.706; **D**, males: R^2=0.0502, p=0.483) and CAE (**E**, females: R^2=0.0981, p=0.321; **F**, males: R^2=0.0802, p=0.372), suggesting that photophobia and anxiety were likely separated, not interdependent measures. As expected, significant correlation was observed between the EPM endpoints % OAT and % OAE both (**G**) in female (R^2=0.466, p=0.0144) and (**H**) male (R^2=0.829, p=0.0000) mice.

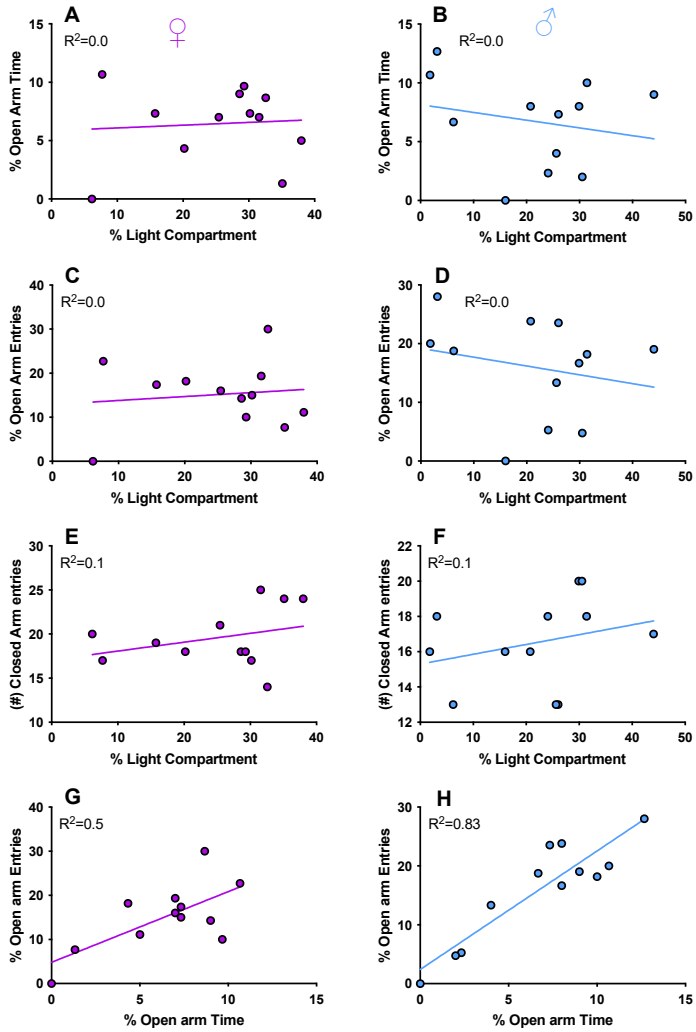

Figure 9. Anxiety parameters do not correlate with photophobia. Linear regression analyses were conducted to determine correlations between photophobia- and anxiety-related variables (N=24, 12 females and 12 males). There was no significant correlation between **(A&B)** % of time spent on the light compartment of the dark-light box (DLB) and the % of time spent on the open arm of the elevated-plus maze (EPM) in female or male mice, respectively. Neither there was significant correlation between **(C&D)** % of time spent on the light compartment of the DLB and the % of entries onto the open arms of the EPM in females and males, as well as between **(E&F)** % of time spent in the light compartment and exploration of the EPM closed arms in female or male mice, respectively. As expected, **G&H** show positive correlations between the % of open arm time and entries both in female and male mice.

Discussion

The neurobiology of migraine involves activation and sensitization of trigeminal meningeal afferent nociceptors, whose cell bodies are in the trigeminal ganglia. These afferents transmit their signal to the brainstem for pain processing [43]. Several preclinical behavioral models have been developed to stimulate dural blood vessels and mimic migraine in humans. A widely used model is meningeal stimulation by direct application of inflammatory compounds [8]. Other methods use the systemic injection of the nitric oxide donor, nitroglycerin [3; 46] or repeated injections of the migraine relief medication sumatriptan, which paradoxically can increase sensitivity to head pain [17]. These etiologically diverse models have been used to understand the pathophysiology of headaches and to develop new therapeutics.

Considerable evidence implicates CGRP in the pathophysiology of migraine [49-51]. Consistently, exogenously administered CGRP, whether delivered directly into the dura through a guide cannula, injected subcutaneously into the periorbital area or delivered by systemic i.p. injections, can induce periorbital mechanical allodynia in laboratory animals [2; 19; 20]. Our findings of CGRP-induced cephalic allodynia are largely in agreement with prior studies [19; 20], as we replicate that a single CGRP exposure induces periorbital allodynia in C57BL/6J mice. However, some differences exist between our findings and others. For example, we observed that i.p. CGRP injection induced allodynia in both female and male mice, whereas dural CGRP administration elicited facial hypersensitivity only in females [2]. Another study showed no sex differences following i.p. CGRP administration of the same CGRP dose that we administered (0.1 mg/kg) [20]. In contrast, we observed that levels of basal allodynic responses in female and male mice were different, with females surprisingly showing less sensitivity to tactile stimulation than males. Differences in the route of CGRP administrations, methods used to assess cephalic allodynia, and acclimation procedures are critical factors that can account for such discrepancies.

One important topic in migraine research is understanding the progression of migraine from an episodic to a chronic disorder. Animal models of chronic migraine-associated pain are critical tools for studying the transition from episodic to chronic migraine, as well as for identifying and screening novel acute and preventive migraine therapies [46; 55]. We approached the problem of developing a model of chronic migraine by performing daily injections of the known migraine trigger CGRP. We found different responses in female and

male mice, with females not always showing acute mechanical allodynia after each CGRP exposure, but displaying a progressively increased basal sensitivity, which reached its maximum after 4 continuous CGRP exposures. The treatment was therefore discontinued after 4 days. This effect was not due to possible associative learning resulting from repeated testing [41; 46], as demonstrated by stable responses observed in vehicle-treated female mice. In contrast, male mice, which once again showed increased levels of basal mechanical sensitivity as compared to females, maintained similar levels of basal allodynia across the 4-day of CGRP treatment; however, CGRP-treated males showed somehow different allodynic responses from the males not receiving CGRP, as the latter decreased mechanical responses following repeated mechanical stimulation across days. Our findings are consistent with a prior study showing that female mice developed increased levels of basal mechanical allodynia more quickly than males following chronic intermittent administration of nitroglycerin [46]. The sexually dimorphic responses we observed following repeated CGRP administration, with females that appear more vulnerable than males in displaying progressively increased headache-like responses, supports the translational relevance of our chronic migraine model.

Except for a few clinical studies where combinations of CBD and Δ^9-tetrahydrocannabinol (THC) were tested [42; 48], there is no available information on the use of CBD as migraine treatment [39]. We show here that CBD, administered to mice prior to a single exogenous CGRP exposure, successfully reversed periorbital mechanical allodynia. Because facial cutaneous allodynia is a symptom that can be found in almost 80% of patients experiencing a migraine attack [10], our data strongly suggests a beneficial effect of CBD in protecting from occurrence of episodic migraine attacks. Notably, the anti-allodynic effect of CBD was observed in mice of both sexes and occurred at doses that did not alter parameters of locomotor activity, thus excluding the possibility that the CBD anti-allodynic effects were secondary to possible non-specific effects on the mouse locomotor behavior. However, no complete dose-response curve for CBD has been performed, so the present data do not allow the estimation of CBD potency in this response. Remarkable CBD effects were also observed in female mice tested on the chronic CGRP model, with both doses of CBD examined being able to reliably prevent acute mechanical allodynia after each CGRP exposure, as well as progressive and sustained basal allodynia. These results suggest that CBD may be effective both in re-establishing the lowered thresholds that result from CGRP-induced changes to meningeal perivascular nociceptors and in preventing the increase in the responsiveness (sensitization) of central neurons responsible for pain processing [9; 10], which is one of the

mechanisms underlying the progression from episodic to chronic migraine [1]. After determining that CBD can be effective in preventing chronic headache we assessed whether CBD could also be effective as a treatment for ongoing migraine-like attacks. We found in female mice that CBD abolished cephalic allodynia even when administered after CGRP, producing an effect similar to that of olcegepant, a CGRP receptor antagonist designed as acute treatment of migraine, whose development was discontinued due to side effects [21]. Collectively, this data suggests that CBD can serve as both a preventive tool and an abortive treatment for migraine. Importantly, exposure of uninjured female mice to CBD over several days demonstrated that CBD can be safely and repeatedly administered with reduced risk of causing medication overuse headache, an effect that can result from repeated exposure to triptans and opioids [4; 17].

CBD effects were then examined in migraine-like symptoms other than allodynia. Facial grimace scale [36] was used as a surrogate readout of spontaneous pain. Increase in facial signs of discomfort following peripherally administered CGRP was reported in a study that did not distinguish between female and male mice [47]. In agreement with that study, we found significantly greater CGRP-induced pain response in mice of both sexes. However, only females showed sustained facial grimace (up to 60 min), which was significantly attenuated by CBD. Systemically administered CGRP also increased light sensitivity in female mice to a larger extent than in males under our experimental conditions, which supports previous evidence of CGRP-induced photophobia [34; 40]. As the DLB is a test commonly used to assess anxiety-like behaviors, and also used to examine light sensitivity, making interpretations of the photophobia test particularly challenging [56], we have taken steps to keep the photophobic and anxious components of the CGRP-induced migraine model experimentally separated and detected. First, we conducted the photophobia assay in conditions of habituation to the DLB testing chambers to avoid the neophobia/anxiety due to novelty. Second, an EPM test was conducted right after the photophobia test using the same mice under the same treatment conditions for both assays to determine whether treatment with CGRP, expected to reduce the time spent into the light compartment of the DLB, also led to anxiogenic-like responses in the EPM. We found differences in the responses to the two tests (e.g., CGRP elicited light-aversive behavior, but not anxiogenic-like behavior in female mice), suggesting that photophobia data cannot be explained in terms of CGRP-induced changes in anxiety-like activity. Furthermore, linear regression analyses didn’t show correlations between photophobia- and anxiety-related variables, which is another indication of proper assessment of both symptoms.

The finding that CBD fails to protect from photophobia is surprising because CBD has proved very effective in managing another sensory amplification of migraine such as cutaneous allodynia. However, the neural circuits underlying migraine-relevant pain circuits and photophobia are different [5] and it is possible that CBD produces effects through the former network, but not through the latter. Because more than 65 molecular targets of CBD have been identified, although many of these appear to be activated at very high concentrations [29], it is hard to establish the exact mechanism through which CBD exerts its therapeutic effects. Within the cannabinoid system, CBD is a CB_1 receptor antagonist, and CB_2 inverse agonist [54]. CBD can also interact with various TRPV channels [29] and is an inhibitor of the fatty acid amide hydrolase, which has been demonstrated to be a potential target for anti-migraine therapy [27]. Furthermore, CBD may also act through the serotonin 1A ($5\text{-}HT_{1A}$) receptors [11], the orphan receptor GPR 55 [38], and PPAR activation [44]. Thus, one of these mechanisms or a combination of them may underlie the CBD's beneficial effects we observed on migraine-like states. In blocking somatic allodynia it is probable that CBD interferes with the mechanism of CGRP-induced peripheral sensitization of nociceptive signaling from the dura mater, which is considered a necessary event in the headache phase of attacks [31].

In conclusion, by modeling acute and chronic migraine-like states in mice, we report that CBD blocks head sensitivity when injected prior to or after the migraine-triggering substance CGRP, suggesting that cannabidiol can be effective both as a preventive tool and as a treatment for headaches. CBD can also be effective in preventing chronic migraine and seems to be devoid of the risk to induce medication overuse headache upon repeated administrations. CBD is also beneficial for other migraine symptoms although failing in blocking photophobia.

Authors Contribution

CS and AC: Conception and design of the study. CS, BF, KTD, GZ, JS, CR, GC and AC: Acquisition and analysis of data. LT and AC: Drafting a significant portion of the manuscript or figures.

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LT is stockholder of Phoenix PharmaLabs. The other authors declare no conflicts of interest.

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2.5 The N/OFQ-NOP receptor as a new target for the development of antimigraine drugs

1. Introduction

Migraine is a pain disorder that affects the vast majority of the adult population worldwide (Vetvik and MacGregor 2017) with a high impact on the individual and society (Global Burden of Disease Cancer Collaboration et al. 2018). Despite migraine being a widespread pathology, its pathophysiology continues to be partially unclear. Many studies demonstrated the central role of the Calcitonin gene-related peptide (CGRP) (Iyengar et al. 2019) and the trigeminal structures in migraine onset (Terrier et al. 2022). These structures are enriched with nociceptin/orphanin FQ (N/OFQ) receptors (NOP) whose activation elicits robust inhibitory effects, thus suggesting a potential role of the N/OFQ pathway in migraine (Targowska-Duda et al. 2020; Ozawa et al. 2015; Neal et al. 2001; Kiguchi et al. 2020; Hou et al. 2003; Witta et al. 2004; Xie et al. 1999).

The current migraine treatments are not satisfying for the majority of migraineurs (Headache Classification Subcommittee of the International Headache Society 2004). The first-line therapy is triptans, which are not well-tolerated by some patients. Other therapeutic approaches see the use of opioid agonists, but because of their side effects, are not recommended (Tepper 2012; Ong and De Felice 2018). Both of these classes promote the development of headaches from drug abuse (MOH), and the first-line therapy is contraindicated in patients with cardiovascular disease (Maassen Van Den Brink and Saxena 2004). A very recent class of treating agents for migraine consists of GCRP receptor antagonists (Gepants) and monoclonal antibodies vs CGRP and its receptor. These showed good efficacy and about 80% of the patients are satisfied with this therapy. Nevertheless, their high cost, and the stringent prescription guidelines, limit their use in most patients (https://www.aifa.gov.it/-/modifica-registri-aimovig-ajovy-ed-emgality-emicrania-).

We hypothesized here that the NOP receptor could be a good target for the development of new antimigraine drugs, given both its inhibitory pathway signaling and its localization at the level of the neuroanatomic structures that are largely involved in the onset of the migraine attack.

We verify our hypothesis by investigating the phenotype of NOP knockout mice (NOP(-/-)). We examine the ability of both CGRP and Nitroglycerin (GTN) to induce mechanical allodynia in the orofacial portion of the mice in an acute model (Demartini et al. 2019; Mason et al. 2017; Rea et al. 2018).

2. Materials and methods

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 (authorization number 73/2021-PR). In vivo studies have been reported following ARRIVE guidelines (McGrath et al. 2010; Kilkenny et al. 2010). All experiments were conducted with mice bred and housed in the University of Ferrara's centralized preclinical research laboratory (LARP) under specific pathogen-free conditions (SPF). All mice were housed in cages with individual ventilation, with a constant temperature of 21 °C, 25% of humidity, and with a 12-hour light/dark cycle, food, and water ad libitum. CD-1 NOP(+/+) and NOP(-/-) males and females mice, aged 2 to 4 months, were used. Genotyping of these mice has been performed in our laboratories, and described in (Holanda et al. 2019).

Drugs - GTN: purchased from Bioindustria LIM s.p.A (Novi Ligure, Italy) dissolved in 5% glucose, 1.5% propylene glycol, and distilled water; used at 10 mg/kg, i.p.

α-rat CGRP: purchased from Merk Life Science (Milan, Italy) and dissolved in PBS; used at doses of 0.01 mg/kg, 0.03mg/kg, and 0.1mg/kg, i.p.

Measurement of periorbital mechanical allodynia – Mice were habituated for 30 minutes to the testing room two days before testing and 30 minutes before the start of the experiment. Male and female mice were tested in separated days. The measure of periorbital mechanical allodynia (PMA) was performed following the up-and-down paradigm described by Dixon (Dixon 1980) and Chaplan (Chaplan et al. 1994). This method is a statistical tool for the determination of the 50% withdrawal threshold in rodents. For cephalic measures, mice were tested inside plexiglass cylinders closed on the upper part by a grid to allow the animal to breathe. The baseline measurement was assessed immediately before the injection of the drugs. PMA was measured in the periorbital region caudal to the eyes and near the midline. Two basal measurements were performed to decrease their withdrawal reflex, and only the second baseline was reported for data analysis. At the test time, seven Von Frey filaments in logarithmic increments of force (0.02, 0.04, 0.07, 0.16, 0.4, 1.0, and 1.4 g) were applied to the periorbital area perpendicular to the skin, with sufficient force to cause slight buckling, and held for approximately 5 s to elicit a positive response. Mice were poked both in the right and left periorbital areas. The stimulation was initiated with the 0.16 g filament. The response was considered positive by the following criteria: mouse stroking the face with its forelimb, withdrawing the head from the stimulus, or shaking the head. The von Frey

filament is removed immediately in case of a positive response. In the presence of a response, a smaller force filament (down) was used in the subsequent test, while the absence of a response led to a heavier force filament (up). After the first breaking point, four more measurements were collected for each mouse or until four consecutive positive or negative responses occurred. The 50% mechanical withdrawal threshold (expressed in g) was then calculated from these scores by using the following equation:

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

The Dixon statistics table provides the k value, and the last filament applied in the XO series is referred to as X_f. 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 (Christensen et al. 2020).

Treatment protocols – All compounds used were administered by intraperitoneal injection (i.p.), in a volume of 10 ml/kg, using a 25G needle. NOP(+/+) and NOP(-/-) mice were injected with GTN 10 mg/kg, with CGRP 0.01 – 0.1 mg/kg or their vehicles. PMA was measured 30, 60, and 120 minutes after drug administration.

Statistical analysis – Data were expressed as mean ± SEM of N animals/group and analyzed with the software GraphPad Prism 9.03 (La Jolla, CA, USA). Data have been shown as threshold across time and as area under the curve (AUC). This allowed the statistical analysis through the two-way analysis of variance (ANOVA) followed by the Tukey post-hoc test. Differences have been considered statistically significant with a p-value < 0.05.

3. Results

NOP receptor genetic blockage increases the sensitivity of mice to GTN effects – 12 male mice and 12 female mice NOP(+/+) and 12 male mice and 12 female mice NOP(-/-), were injected i.p. with GTN 10 mg/kg. GTN induced PMA both in NOP(+/+) and NOP(-/-) male mice. Male mice NOP(-/-) mice showed increased PMA values compared to NOP(+/+) mice. GTN 10mg/kg failed to significantly affect NOP(+/+) female mice. In contrast, it significantly increased PMA in NOP(-/-) female animals (Figure 1).

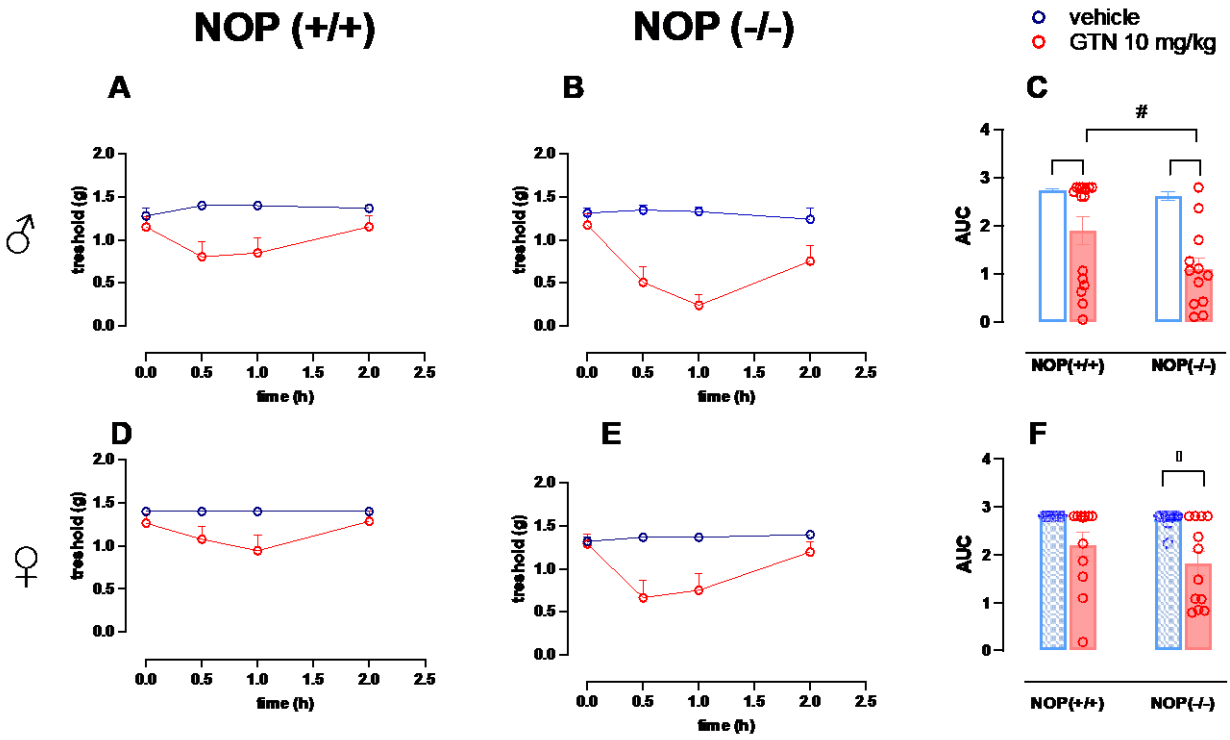

Figure 1. Effect of the i.p. administration of GTN in NOP(+/+) and NOP(-/-) male (panel A, B, and C) and female (panel D, E, and F) mice. The sensitivity threshold (g) was assessed 30 minutes, 1, and 2 hours following acute administration of either 10 mg/kg of GTN or its vehicle. Panels A, B, D, and E show the time course of the effect of GTN, while panels C and F show the same data expressed as AUC. Dara are expressed as mean ± s.e.m., n = 12 mice/group. Two-way ANOVA (treatment x genotype) followed by Tukey's multiple comparison tests revealed an effect of treatment ($F(1,46) = 31.00$, male; $F(1.44) = 17.83$, female), and genotype ($F(1.46) = 4.80$, male; $F(1.44) = 1.56$, female). *$p<0.05$, vs vehicle. #$p<0.05$ vs NOP(+/+) mice.

NOP receptor genetic blockage increases the sensitivity of mice to CGRP effects – Different doses of CGRP, from 0.01 to 0.1 mg/kg have been tested in NOP(+/+) and NOP(-/-) mice. CGRP 0.1 mg/kg induced increased PMA in both male and female NOP(+/+) and NOP(-/-) mice (figure 2). No differences were measured at this high dose between the two genotypes. When the lower CGRP doses were tested, some differences between NOP(+/+) and NOP(-/-) mice were recorded. In fact, CGRP 0.01 mg/kg induced PMA only in NOP(-/-) male mice but not in NOP(+/+) male mice (Figure 2 panel A, B and C). In female mice, CGRP 0.03 mg/kg induced higher PMA in NOP(-/-) mice than in NOP(+/+) mice (Figure 2 panel D, E and F).

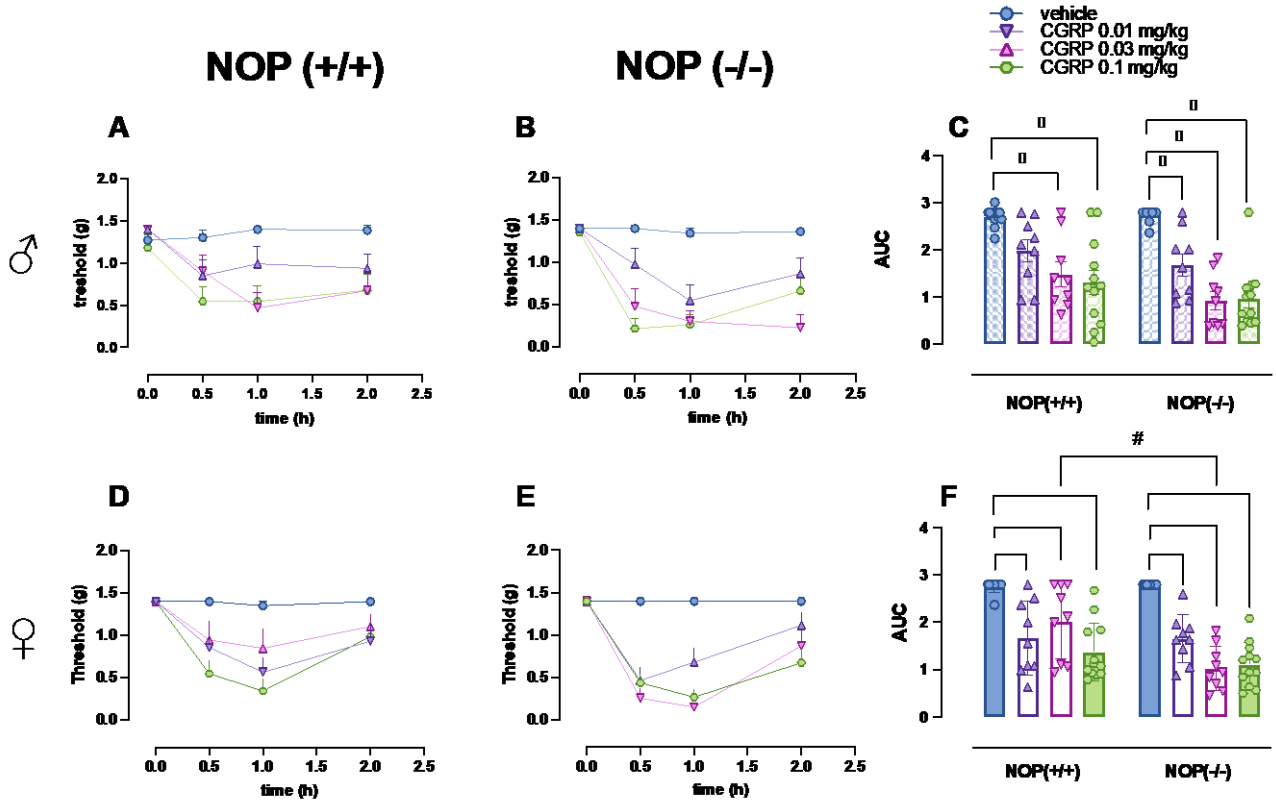

Figure 2. effect of i.p. administration of CGRP 0.01, 0.03 and 0.1 mg/kg i.p. in NOP(+/+) and NOP(-/-) male (panel A, B, C) and female (panel D, E, F) mice. Sensitivity threshold (g) were assessed 30 minutes, 1, and 2 hours following acute administration of either CGRP or its vehicle. Panels A, B, D and E show the time course of the effect of CGRP, panels C and F show the same data expressed as AUC. Data are expressed as mean ± s.e.m., n = 12 mice/group. In panel C, two-way ANOVA (treatment x genotype), followed by Tukey's multiple comparison test, revealed an effect of treatment ($F(3.74) = 28.52$), genotype ($F(1.74) = 4.208$), and their interaction ($F(3, 75) = 3{,}807$). In panel F two-way ANOVA (treatment x genotype), followed by Tukey's multiple comparison tests, revealed an effect of treatment ($F(3.75) = 38.49$), and genotype ($F(1.75) = 7.143$). *$p<0.05$ vs vehicle, #$p<0.05$ vs NOP(+/+).

4. Discussion

In the present study the phenotype of NOP(+/+) and NOP(-/-) mice has been investigated in two commonly used mouse migraine models: GTN-induced migraine and CGRP-induced migraine. We demonstrated that in NOP(-/-) there is a tendency to be more sensitive to the effects of both GTN and CGRP, suggesting a role of the NOP receptor in migraine susceptibility.

The systemic injection of NO donors, i.e. GTN, is largely used by the scientific community to induce migraine-like pain in rodents (Afridi et al. 2005; Maniyar et al. 2014; Di Clemente et al. 2009; de Tommaso et al. 2004; Perrotta et al. 2011; Buzzi and Tassorelli 2010; Tassorelli and Joseph 1995; Tassorelli et al. 1999, 1997, 2005; Bates et al. 2010; Greco et al. 2015, 2018a, 2018b; Knapp et al. 2017; Demartini et al. 2017; Akerman et al. 2019; Marone et al. 2018). The mechanisms by which GTN-induced headache are still not completely understood. They include the activation of the soluble guanylyl cyclase, with consequent vasodilatation (Ben Aissa et al. 2018), and other cGMP-independent actions, i.e. oxidative stress, increased expression of neuronal and inducible nitric oxide synthase and nuclear factor kappa B in the trigeminal area with consequent activation of trigeminal fibers, CGRP release, neurogenic inflammation and peripheral and central sensitization (these mechanisms have been reviewed by (Demartini et al. 2019). What is important is the translational value of this migraine model. In fact, GTN has the ability to induce headaches with migraine-like features in humans, and its administration is clinically used as an experimental migraine model. Interestingly GTN induced headache attacks fulfill the diagnostic criteria of the International Headache Society only in migraineurs or, in some studies, in healthy subjects with a positive family history of migraine (Demartini et al. 2019). The translatability of the GTN model of migraine has been addressed in an elegant study by Akerman et al. (Akerman et al. 2019) that parallels the ability of GTN to induce cranial allodynia (considered a migraine sign) in migraine patients and rats. This study demonstrated that GTN elicits cranial allodynia both in patients and in rats and that triptan drugs can abort this effect in both humans and rats. This study supports the use of GTN as a migraine-like trigger and the evaluation of cranial allodynia as a migraine sign in rodents. Of note, PMA, measured with von Frey filaments, is now considered in rodents a major migraine sign, as demonstrated by the high number of research articles using this approach (De Logu et al. 2019, 2022; Marone et al. 2018; Dalenogare et al. 2022; Akerman et al. 2019; Pradhan et al. 2014; Bates et al. 2010; Moye et al. 2019; Bertels et al. 2022; Avona et al. 2021). This evidence prompted us to select GTN as a migraine trigger and PMA as a migraine sign to evaluate the involvement of the NOP receptor in the onset of a migraine

attack. Under our experimental conditions, GTN 10 mg/kg caused PMA in male wild-type but not in female wild-type mice. The results obtained in male mice agree with a large amount of scientific literature demonstrating that 10 mg/kg is the GTN dose able to induce PMA in mice with a similar time course of the effect (Bates et al. 2010; Gölöncsér and Sperlágh 2014; Tipton et al. 2016; Marone et al. 2018). Unexpectedly female mice did not develop PMA after GTN administration. The comparison of this result with the scientific literature is difficult since the majority of the studies have been performed with males and with C57BL/6 mice. It is known that the GTN sensitivity can vary among different mice strains and that sex is also an important variable. Anyway, a parallel study performed in our laboratories using the same GTN batch demonstrated the ability of GTN to induce PMA in female C57BL/6 mice. Thus the inactivity of GTN in the female CD-1 mice, used in this study, is probably due to an interaction strain x genotype rather than a pure sex effect. Both male and female NOP(-/-) mice showed increased PMA after GTN administration compared to NOP(+/+) mice, suggesting that the absence of NOP makes mice more sensitive to the migraine-like effects of GTN. Thus, it can be hypothesized that N/OFQ is released in peripheral and brain areas involved in migraine onset after migraine trigger and that the activation of the NOP receptor in these structures reduces migraine-like pain. To corroborate this hypothesis the phenotype of NOP(-/-) has been also investigated in a different model that uses CGRP as a migraine trigger. Considering the involvement of CGRP in the migraine process and the ability of CGRP to induce migraine attacks in humans (Ashina et al. 2013), animal models of migraine induced by the injection of CGRP have been developed. CGRP administration by peripheral and central routes has been demonstrated to induce different signs of migraine in rodents, including PMA (Wattiez et al. 2019; De Logu et al. 2022; Avona et al. 2019). In agreement with this scientific literature, under our experimental conditions, CGRP (0.1 mg/kg, i.p.) induced a strong PMA both in male and female wild-type mice. No differences were detected between NOP(+/+) and NOP(-/-) mice. Anyway, to avoid the interference of confounding floor effects, some experiments have been performed using CGRP lower doses. In male mice, the CGRP dose of 0.01 mg/kg elicits PMA in NOP(-/-) mice but not in NOP(+/+) mice. Similarly, CGRP 0.03 mg/kg causes higher PMA in NOP(-/-) than in NOP(+/+) mice. Thus, NOP(-/-) animals displayed higher sensitivity to CGRP migraine-like effects than NOP(+/+) animals. This result parallels those obtained with GTN injection and further supports the hypothesis that the NOP receptor is activated during migraine trigger exposure and its activation mitigates migraine symptoms. This view suggests the utility of NOP agonists for the treatment of migraine. This hypothesis is sustained by some behavioral studies performed in mice. The selective NOP non-peptide

agonist Ro-656570 was able to reduce pain behaviors induced by the injection of formalin in the orofacial region (Rizzi et al. 2017). Additionally, the NOP non-peptide agonist Ro 64-6198, was able to revert the GTN-induced PMA in a dose-dependent manner (Targowska-Duda et al. 2020). Of note, the use in these studies of small molecules of NOP agonists, given systemically and able to reach the brain, did not provide information regarding the NOP receptors involved in these effects i.e. peripheral vs central receptors. Regarding the mechanisms by which NOP receptor activation modulates migraine, some speculation can be based on literature findings. N/OFQ and its receptor are present in neuroanatomical structures important for the onset of migraines, such as the trigeminal ganglion and the trigeminal caudal nucleus, both in rodents (Neal et al. 1999a; Xie et al. 1999; Targowska-Duda et al. 2020; Neal et al. 1999b) and in humans (Xie et al. 1999; Witta et al. 2004; Hou et al. 2003; Mørk et al. 2002). In these regions, they colocalize in part with CGRP (Targowska-Duda et al. 2020; Toll et al. 2021). In these areas, the NOP activation produces inhibitory effects. Studies done on neuronal cultures of trigeminal rat ganglions demonstrated that the activation of NOP causes membrane hyperpolarization (Connor and Christie 1999) and inhibition of CGRP release induced by different stimuli (Capuano et al. 2009, 2007). Additionally, N/OFQ has been shown to produce inhibitory effects in the trigeminal caudal nucleus's neurons (Wang et al. 1996; Bartsch et al. 2002). Thus, it can be supposed that, during a migraine attack, NOP receptors activation controls migraine-like pain and signs by acting both at the trigeminal ganglion level, inhibiting neuronal excitation and CGRP release, both in the spinal trigeminal nucleus by reducing neuronal activity. The lack of this inhibitory control exerted by NOP stimulation is responsible for the increased sensitivity of NOP(-/-) mice to GTN and CGRP effects. This is the very first study that tries to evaluate the NOP(-/-) phenotype in a model of migraine. Anyway, some studies evaluating the phenotype of NOP(-/-) mice in different pain models are available. NOP(-/-) mice were reported to be more sensitive to the formalin nociceptive effect, both in the paw (Rizzi et al. 2006; Depner et al. 2003) and in the trigeminal area (Rizzi et al. 2006, 2017). Similar results were obtained with NOP(-/-) rats (Rizzi et al. 2011). On the other hand, no differences between NOP(+/+) and NOP(-/-) mice were detected in models of acute pain such as the tail-withdrawal test (Di Giannuario et al. 2001; Nishi et al. 1997). Similarly, no differences were recorded between NOP(+/+) and NOP(-/-) rats in the Hargreaves test (Rizzi et al. 2011). Overall, these studies support the involvement of the N/OFQ-NOP system in the modulation of chronic and inflammatory pain but not nociceptive pain. Of note, the higher sensitivity of NOP(-/-) mice to the formalin

effects in the trigeminal area (Rizzi et al. 2006, 2017) can be considered in line with their propensity to develop higher PMA following GTN or CGRP administration.

In conclusion, this study straight from the previous studies reported by (Targowska-Duda et al. 2020) and by Rizzi et al. 2017 and suggests NOP agonists as new possible anti-migraine drugs. Shortly, further studies are necessary to validate this therapeutic target. I.e. the studies published and the present research, performed with acute migraine models, encourage the use of NOP agonists as acute abortive migraine treatments. Nothing is known about the possible NOP involvement in the migraine progression to chronic disease. Another point to be addressed is related to the population of NOP receptors important for migraine modulation. Peptide NOP ligands, that do not cross the blood-brain barrier, can represent useful tools to answer this question.

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3. GENERAL CONCLUSIONS

The work presented in this Ph.D. thesis arises from the urgency for the identification of new drugs capable of treating or preventing migraine. A deep analysis of the scientific literature suggested the receptors kappa, NOP/mu, and TRPA1 as proteins involved in migraine disease, as well as CBD as a natural product worth to be investigated for its anti-migraine properties. Thus, I decided to address all these topics to provide the scientific community with new methodologies, compounds, and evidence useful to speed up the development of new anti-migraine drugs. In summary, my research work led to i) the setup and validation of new methodologies and protocols to characterize in vitro kappa receptor ligands; ii) the identification of a potent NOP/mu agonist and two potent TRPA1 antagonists immediately available for the in vivo evaluation; iii) the demonstration that CBD produces anti-migraine effects in a mouse model of migraine, thus supporting its evaluation in clinical trials; iv) the demonstration of the involvement of the NOP protein in migraine, that support further research aimed to the evaluation of standard and new NOP agonists in pre-clinical migraine models.

More in detail, one goal of this work was the set-up and pharmacological validation of a platform of *in vitro* assays to characterize ligands for the kappa opioid receptors. This includes calcium mobilization in cells expressing chimeric G proteins, the DMR label-free assay, and a BRET assay that allows measurement of receptor interaction with G protein and β-arrestin 2. All the assays have been pharmacologically validated using a battery of standard kappa ligands. In the frame of this study, the two new dynorphins A derivatives have been characterized: PWT2-Dyn A and Dyn A-palmitic. These compounds behaved as potent full kappa agonists. Actually, despite the utility of such compounds in some conditions, like pain and pruritus, the scientific literature doesn't suggest kappa agonists as antimigraine agents, while kappa antagonists seem to be promising in the treatment of stress-induced migraine (Xie et al. 2017; Kopruszinski et al. 2022; Bernard et al. 2019; Watanabe et al. 2022).

Anyway, thanks to this work, a plethora of assays to identify new kappa antagonists, in collaboration with medicinal chemistry groups, are now available in our laboratories. Another objective of my research activity was the *in vitro* pharmacological characterization of new mixed NOP/mu peptide agonists. This activity has been done in close collaboration with Prof. Guerrini's medicinal chemistry research group (University of Ferrara). Specifically, 31 new compounds with the general sequence [Tyr/Dmt^{1}Xaa^{5}]N/OFQ(1-13)NH2 have been synthesized and investigated through the calcium mobilization and the DMR assay, using cells stably expressing the NOP, mu, delta, and kappa receptors. The main

success of this study was the identification of [$Dmt^{1,5}$]N/OFQ(1-13)-NH_2, a potent mixed NOP/mu agonist, that represents the most potent dual NOP/mu receptor peptide agonist so far described. Of note, the in vivo activity of this compound has been already demonstrated, in the frame of this study, in a model of cough. This peptide may likely be a new antimigraine therapy, as suggested by the literature (Kiguchi et al. 2022). Of note, the peptide nature of this compound leads to some advantages (i.e. high selectivity for the target with a very low probability of off-target side effects, possibility to act in the periphery without reaching the CNS) but also to some pharmacokinetic limitations that should be taken into account to evaluate the compound in vivo. Additionally, in collaboration with Prof. Preti's medicinal chemistry research group (University of Ferrara), I identified and pharmacologically characterized in vitro new TRPA1 antagonists. The TRPA1 receptor seems to be strongly involved in the onset of migraine (Edelmayer et al. 2012; Nassini et al. 2012; Marone et al. 2018). All the compounds synthesized and tested were analogs of the standard TRPA1 antagonist DHC200. The chemical formulae of the new compounds are not shown in this work because of industrial interests. Among these, I identified DHC236 and DHC277 as TRPA1 pure antagonists, three times more potent than DHC200, which could be promising antimigraine drugs. Importantly, to predict the effectiveness of both [$Dmt^{1,5}$]N/OFQ(1-13)-NH_2 and the two DHC200 analogs as antimigraine drugs, is mandatory to test them in vivo, in pre-clinical migraine models. These experiments will be performed in the near future. Of note, thanks to the work I performed during the past years, two murine migraine models are now available and routinely used in our laboratories.

In fact, in parallel with these *in vitro* studies, I also performed *in vivo* experiments with the aim of developing two different models of migraine in mice using two migraine triggers: CGRP and GTN, and subsequently evaluating, as signs of migraine-like pain, the periorbital mechanical allodynia (PMA) through the use of manual Von Frey filaments.

I evaluate CBD in a mouse model of migraine induced by CGRP. Specifically, CBD has been assessed for its ability to prevent and block acute migraine attacks and to prevent the development of chronic migraines. I demonstrated that CBD, administered both prior and after to a single CGRP exposure, reversed PMA. Moreover, CBD is resulted to be effective in preventing chronic headaches. Since facial cutaneous allodynia is a symptom that can be found in almost 80% of patients suffering from a migraine attack (Burstein et al. 2000), these results suggest a beneficial effect of CBD in protecting and reversing the occurrence of episodic migraine attacks. CBD already entered several clinical trials for several conditions (i.e. epilepsy, anxiety, addiction, and pain) (Sholler et al. 2020) a CBD oral formulation exists (Epidiolex®) and has been recently approved by FDA and EMA for Lennox-Gastaut

and Dravet syndromes, moreover, CBD resulted well tolerated by adults (Iffland and Grotenhermen 2017). These considerations suggest the feasibility of the translation of the results of my preclinical studies to migraine patients.

Finally, I investigated *in vivo* the involvement of the NOP receptor in migraine by studying the phenotype of mice knockout for the NOP receptor in two experimental migraine models: GTN-induced migraine and CGRP-induced migraine. As a result of this study, I demonstrated that NOP(-/-) mice are more sensitive to the effects of both GTN and CGRP, suggesting the role of the NOP receptor in migraine onset. This result supports the hypothesis that the NOP receptor is activated during migraine trigger exposure and its activation mitigates migraine symptoms, corroborating the utility of NOP agonists for the treatment of migraine.

In conclusion, this Ph.D. project provides the scientific community with new research lines to better understand migraine and to identify and develop new drugs. Thanks to the collaboration between different medicinal chemistry teams and different pharmacology units, the described work generated new methodologies, chemical entities, and pre-clinical evidence that will likely facilitate in the near future the identification and/or development of innovative antimigraine therapeutics.

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5. OTHER COLLABORATIONS

Full papers

1. Wtorek, K.; Ghidini, A.; Gentilucci, L.; Adamska-Bartłomiejczyk, A.; Piekielna-Ciesielska, J.; Ruzza, C.; **Sturaro, C.**; Calò, G.; Pieretti, S.; Kluczyk, A.; McDonald, J.; Lambert, D.G.; Janecka, A. *Synthesis, Biological Activity and Molecular Docking of Chimeric Peptides Targeting Opioid and NOP Receptors*. *Int. J. Mol. Sci.* **2022**, *23*, 12700. DOI: 10.3390/ijms232012700. PMID: 36293553
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3. Illuminati D, Fantinati A, De Ventura T, Perrone D, **Sturaro C**, Albanese V, Marzola E, Cristofori V, Oble J, Poli G, Trapella C. *Synthesis of 2,6-Dimethyltyrosine-Like Amino Acids through Pinacolinamide-Enabled C-H Dimethylation of 4-Dibenzylamino Phenylalanine*. J Org Chem. 2022 Mar 4;87(5):2580-2589. DOI: 10.1021/acs.joc.1c02527. Epub 2022 Feb 9. PMID: 35138099
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Paper submitted for publication:

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Abstract:

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