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Review

Nitroglycerin as a model of migraine: Clinical and preclinical review

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ABSTRACT

Migraine stands as one of the most disabling neurological conditions worldwide. It is a disorder of great challenge to study given its heterogeneous representation, cyclic nature, and complexity of neural networks involved. Despite this, clinical and preclinical research has greatly benefitted from the use of the nitric oxide donor, nitroglycerin (NTG), to model this disorder, dissect underlying mechanisms, and to facilitate the development and screening of effective therapeutics. NTG is capable of triggering a migraine attack, only in migraineurs or patients with a history of migraine and inducing migraine-like phenotypes in rodent models. It is however unclear to what extent NTG and NO, as its breakdown product, is a determinant factor in the underlying pathophysiology of migraine, and importantly, whether it really does facilitate the translation from the bench to the bedside, and vice-versa. This review provides an insight into the evidence supporting the strengths of this model, as well as its limitations, and shines a light into the possible role of NO-related mechanisms in altered molecular signalling pathways.

Introduction

What is nitroglycerin and its physiological effects?

Nitroglycerin (NTG, Fig. 1**A**) is a highly permeable, lipophilic, organic nitrate that has been extensively used as a model of migraine. It is its action as a nitric oxide (NO) donor that is thought to mediate its migraine-inducing effects. While the exact mechanism of NTG bioactivation and release of NO is not clear it is thought that mitochondrial aldehyde dehydrogenase (ALDH2, mtALDH) is involved in catalysing the denitration and reduction of NTG to NO (Chen et al., 2005, Chen and Stamler, 2006). While the conversion of NTG into NO was initially thought to be primarily localised in the vascular walls to uniquely modulate vascular tone, the presence of NO in other tissues uncovered key modulatory roles in both the nervous system and in inflammatory responses (Moncada and Higgs, 2006). NO therefore represents a key neurotransmitter with widespread actions in the central nervous system (CNS) due to its high permeability and diffusion properties to nearby neural and glial structures (Dawson et al., 1994). Denitrification from nitroglycerin (NO3) to NO takes place via a four-step process (NO3− → NO2− → NO → N2O → N2), with NO2 and NO3 being the source of NO storage in both mammals and bacteria. Bacteria in the oral cavity and microbiome are responsible for the initial NO3 – NO2 conversion, leaving NO2 available to be transformed at a cellular level through both enzymatic and non-enzymatic reactions (Koch et al., 2017) (Fig. 1**B**).

Endogenously, NO production involves three different NO synthase (NOS) isoforms to form a dimer and catalyse the oxidation of L-arginine into L-citrulline. The three NOS isoforms; neuronal NOS (nNOS, NOS1 or NOSI), inducible NOS (iNOS, NOS2 or NOSII) and endothelial NOS (eNOS, NOS3 or NOSIII) (Fig. 1**B**), are named based on the tissue in which they were initially identified. However, they are all extensively localised in the periphery and CNS. Each has been implicated in the generation of NO from NTG, based on their tissue localisation (Reuter et al., 2001, Reuter et al., 2002, Bonini et al., 2008, Dieterle et al., 2011) and iNOS has been suggested to release larger amounts of NO, compared to eNOS (Wilcox and Fuller, 1991). Despite this, recognising the potential mechanistic differences between the three isoforms has proven crucial to developing clinically translatable treatments. For instance, while the use of non-selective NOS inhibitors initially showed efficacy in animal models of septic shock; this was discontinued clinically due to an increased mortality rate (Lopez et al., 2004), suggesting NOS isoform selectivity is preferable to avoid side effects such as hypertension (Alderton et al., 2005). This is perhaps unsurprising given the wide range of functions of NOS; from modulating vascular tone and blood pressure (Rees et al., 1989), inhibiting platelet aggregation, regulating inflammatory responses (Moncada and Higgs, 2006) and mitochondrial

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respiration (Moncada and Erusalimsky, 2002), to centrally activating NMDA receptors in both neurons and glia, and thus increasing glutamate release (Garthwaite et al., 1988). For a more detailed review of NOS-related mechanisms we refer the reader to a recent review article (Pradhan et al., 2018). In the context of migraine, as will be discussed further, NOS inhibition has indeed become an innovative therapeutic approach (Barbanti et al., 2014).

Nitroglycerin and headache

The association of NTG with headache has been known for over 150 years since its invention by Ascanio Sorbrero, who reported intense headache with handling. More recently, with its use in patients with angina pectoris and myocardial infarction, established at the end of the 19th Century, headache was confirmed as a commonly reported side effect (Dalsgaard-Nielsen, 1955, Sicuteri et al., 1987, Iversen et al., 1989). Intravenous (IV) NTG induces a mild-moderate, bifrontal, and throbbing-like headache within minutes, in both migraineurs and non-migraineurs, making it a reliable model of vascular headache (Olesen et al., 1993, Marsh and Marsh, 2000). This headache has been associated with NTG's potent vasodilatory effects, which are thought to be mediated by the conversion of NTG into NO in the endothelial layer of vascular wall (Tassorelli et al., 1999).

However, while NTG is able to trigger a mild-moderate headache in non-migraineurs, it can also trigger a headache attack with migraine-like features exclusively in migraineurs (Peters, 1953, Dalsgaard-Nielsen, 1955, Hansen and Drewes, 1970, Thomsen et al., 1994a), or subjects with a family history of migraine (Sances et al., 2004). This migraine-like headache attack can initiate within 45 min of NTG administration or be delayed up to 4–5 h, after the initial moderate headache has resolved. Independent of route of administration; intravenous, sublingual, or oral, NTG induces a migraine-like headache attack in 50–80 % of patients (Thomsen et al., 1994b), and importantly, also triggers the occurrence of common premonitory and associated symptoms (Afridi et al., 2004, Maniyar et al., 2014). The temporal nature and clinical features of these NTG-mediated posterior migraine attacks suggest a downstream mechanism of action within the CNS.

Clinical and preclinical presentation of NTG-evoked migraine-like attacks

Clinical presentation: The ability of NTG to trigger a migraine-like attack (based on modified ICHD criteria; Table 1), often described as identical to spontaneously occurring migraine attacks, makes it a reliable clinical model to investigate the underlying pathophysiological mechanisms during an attack. Indeed, of all the pharmacological approaches to trigger migraine-like headache in patients, NTG has been the most studied and is perhaps the most reliable and reproducible (Ashina et al., 2013, Ashina et al., 2017). NTG also mediates clear activation of central migraine-associated pain structures, based on fMRI and

Table 1
The modified migraine criteria for migraine-like attack in experimental studies.

Diagnose	Migraine-like attack after pharmacological provocation
Diagnostic Criteria	1) Headache fulfilling ICHD-II criteria C and D for migraine without aura **C)** Headache has at least two of the following characteristics 1). Unilateral location 2). Pulsating quality3). Moderate or severe pain intensity (>4 on VRS)4). Aggravation by cough (in-hospital phase) or causing the avoidance of routine activity (out-hospital phase) (e.g. walking or climbing stairs) **D)** During headache at least one of the following 1). Nausea and/or vomiting 2). Photophobia and phonophobia 2) Headache described as mimicking usual migraine attack and treated with triptan/aspirin

Taken and adapted from (Headache Classification Committee of the International Headache Society, 2004, Ashina et al., 2013). VRS – verbal rating scale.

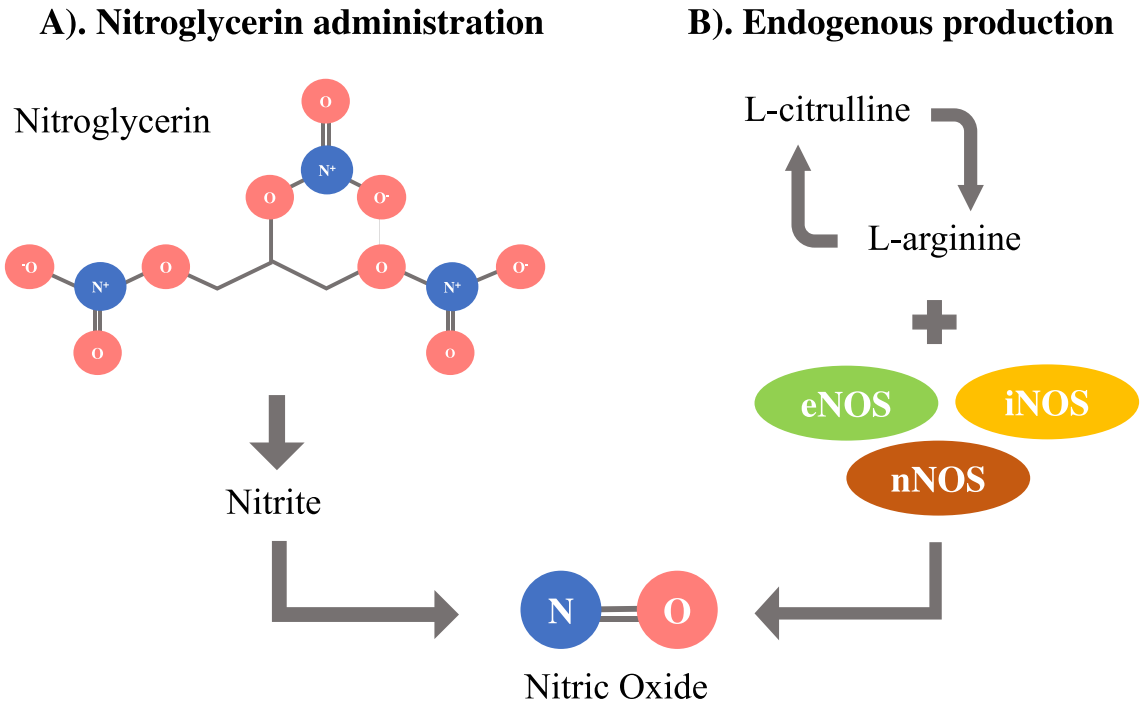

Fig. 1. A.) Nitroglycerin (NTG) administration and its molecular structure. Nitric oxide (NO) donors, such as NTG, are converted into nitrites, through a two or three-step reaction, where they are then converted into NO. **B. Endogenous production of Nitric Oxide (NO).** Endogenously produced NO is formed by the conversion of L-citrulline into L-arginine through arginine synthase (AS). In the presence of O_2 and NO synthase (eNOS, iNOS and/or nNOS), L-arginine is then converted into NO (Adapted from Ghalayini, 2004). There is evidence that reduction of NTG to NO does involve NOS but the exact mechanism is not clear (Reuter et al., 2001, Reuter et al., 2002, Bonini et al., 2008, Dieterle et al., 2011).

physiological studies, in the upper cervical spinal cord and trigeminal nuclei (Perrotta et al., 2011), as well as in the hypothalamus, midbrain and pontine regions, amongst other key nuclei (Afridi et al., 2004, Maniyar et al., 2014). However, NTG has no effects on normal extracranial somatosensory nociceptive blink reflex responses (Thomsen et al., 1996, Kowacs et al., 2003). Importantly, these migraine-like attacks are accompanied by commonly reported premonitory and associated symptoms, including extracranial cutaneous allodynia, nausea, photophobia and phonophobia, and pain exacerbated by movement (Afridi et al., 2004), which, given the short half-life of NTG in blood of around 3–4 min (Divakaran and Loscalzo, 2017), suggests a potentially central mechanism of action. NTG-triggered migraine also causes increased levels of migraine-related neurotransmitters, such as calcitonin gene-related peptide (CGRP), in the blood plasma of samples taken from jugular vein, indicative of changes in the cranial circulation (Juhasz et al., 2003). Finally, NTG-triggered attacks can be aborted by common anti-migraine drugs such as sumatriptan and aspirin (Juhasz et al., 2005, Akerman et al., 2019). Sumatriptan was also found to normalise the increase in transmitter levels (Juhasz et al., 2005); suggesting a common mechanism of action to spontaneous migraine attacks (Fullerton et al., 1999). Together, these data support a strong overlap of NTG-mediated migraine-like headache and patients' spontaneous migraine.

Preclinical translation: Importantly, this non-invasive experimental clinical approach can be readily translated to animals to generate both acute and more chronic preclinical migraine-like models.

Acute NTG model

The first studies in rodents demonstrate that a single systemic (subcutaneous, SC) injection of a relatively high dose (10 mg/kg) of NTG in rats mediates strong Fos immunoreactivity (indicative of neuronal activation) in many brainstem and diencephalic areas related to migraine-like mechanisms, including the medullary trigeminal nucleus caudalis (TNC) region and periaqueductal gray (PAG) (Tassorelli and Joseph, 1995). It also causes increased NOS in the TNC (Pardutz et al., 2004). Subsequently, electrophysiological studies demonstrate that an acute dose of NTG mediates activation and sensitisation of both primary afferent (trigeminal ganglion) (Zhang et al., 2013) and central trigeminocervical neurons (Akerman et al., 2019); the underlying mechanism thought to be involved in many nociceptive craniofacial migraine symptoms (Fig. 2). Importantly, these responses are aborted by triptans and newly approved migraine drug classes, CGRP receptor antagonists and 5-HT1F agonists (Akerman et al., 2019; Akerman et al., 2021a). These studies also confirm that in naïve rats that lower doses (1 mg/kg, SC) are largely ineffective.

Studies in conscious mice reveal that acute systemic NTG (10 mg/kg) also induces migraine-like periorbital (Moye et al., 2019) and hind-paw (Bates et al., 2009, Pradhan et al., 2014a) hypersensitivity, measured by probing with von Frey filaments. This is indicative of migraine-like craniofacial and extracephalic allodynia in patients (Burstein et al., 2000, Burstein et al., 2010) and its underlying mechanism, central second-order sensitisation of trigeminal neurons and third-order sensitisation of trigeminothalamic neurons (Burstein et al., 1998, Burstein et al., 2010). Importantly, this correlates in patients, with one study reporting that of those patients that report extracranial allodynia during their migraine attacks, 65 % also report allodynia alongside migraine-headache during NTG-triggered attacks (Akerman et al., 2019). Both migraine headache and associated extracranial allodynia were aborted by therapeutics, similar to rodent studies, representing a crucial translational outcome measure. How migraine treatments can abort allodynic symptoms is still debated, based on differing migraine philosophies (Burstein et al., 2015, Goadsby et al., 2017), however, dural vascular, trigeminal afferent and second-order projection neurons, as well as other central sites are all possible loci of action.

In genetic mouse models, such as mice carrying the human CK1δ-T44A missense mutation, which is thought to be responsible for migraine and familial advanced sleep phase syndrome (FASPS), the NTG dose can be titrated down, with 5 mg/kg being sufficient to mediate hind-paw mechanical and thermal hypersensitivity (Brennan et al., 2013). There is currently no data on the response of the FASP patient population to NTG. In another patient population where a mutation in the potassium channel, TRESK, is implicated in migraine and nociception, TRESK knockout mice demonstrate an exaggerated response to

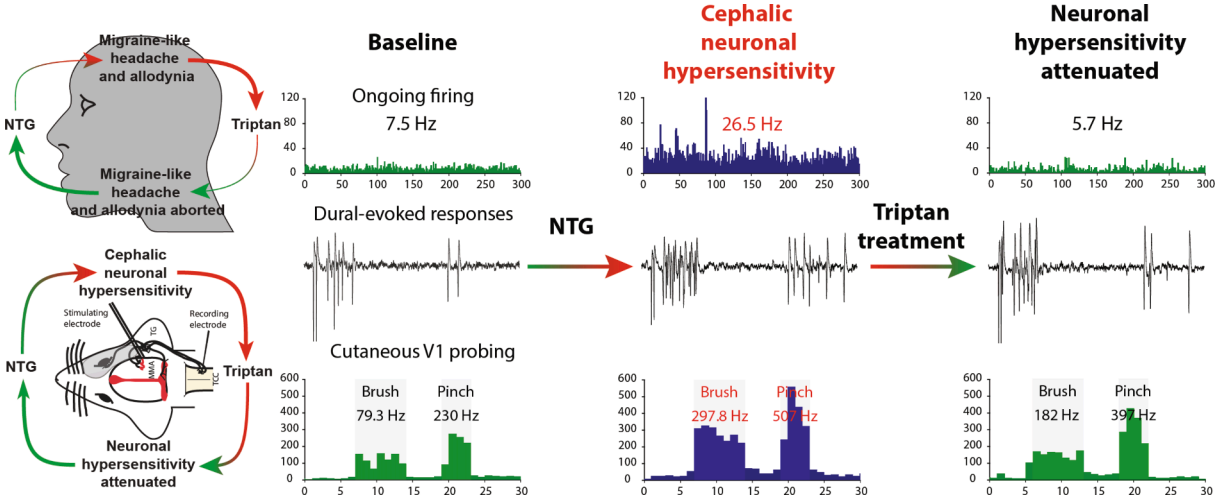

Fig. 2. Overview of the translational nature of nitroglycerin (NTG) as a model of migraine in experimental clinical and preclinical research: Left Panel (top). Schematic representation of the effects of NTG as a human migraine model, mediating migraine-like headache, allodynia, and other migraine associated features that are aborted by treatment with acute migraine therapies, including triptans. Left panel (bottom). The preclinical neuronal correlate of this migraine-like representation in rodents, where NTG mediates intracranial and extracranial neuronal hypersensitivity of trigeminovascular neurons that is aborted by triptan treatments. The three right columns represent an example of electrophysiological recording of a trigeminocervical neuron in rat, with ongoing spontaneous firing (top), a neuronal response to dural electrical stimulation (middle), and response to somatosensory probing of the cutaneous periorbital region to both innocuous brush and noxious pinch stimulation (bottom). Left column represents responses at baseline. The middle column represents responses after nitroglycerin (10 mg/kg, SC) where central trigeminovascular neurons become activated (increase in ongoing firing) and sensitized (hypersensitive neuronal responses to intra and extracranial stimulation) after 60–90 min. After sensitization is established treatment with a triptan aborts these increased neuronal responses, taking them back to near baseline levels. This figure is adapted and modified with permissions from (Akerman et al., 2019).

NTG (10 mg/kg) compared to controls (Pettingill et al., 2019). Lower doses have not been studied. In other behavioural pain assays NTG also mediates hypersensitive responses in the thermal Hargreaves' (Bates et al., 2009) and hotplate (Farkas et al., 2016) tests, cold allodynia test (Kim et al., 2018), and using an operant Orofacial Pain Assessment Device (OPAD) (Anderson et al., 2013). Here, NTG mediates orofacial thermal hypersensitivity **(**Fig. 3**)** (Sureda-Gibert et al., 2018). Other migraine-like behaviours such as increased anxiety-like behaviours (Askari-Zahabi et al., 2022) and locomotor deficits (Pourrahimi et al., 2021) have also been identified, and importantly, can be aborted or normalised through commonly used anti-migraine drugs **(**Table 1**)**.

Chronic NTG models and priming

NTG has also been utilised to develop a chronic or persistent model of migraine. Here, the premise is to use repeated injections of NTG (10 mg/kg) over many days to replicate more frequent migraine attacks. In this model NTG is administered systemically five times over nine days (every other day) and it mediates persistent basal hind-paw and *peri*-orbital hypersensitivity, particularly in females that return to baseline levels around day 15, approximately 6–7 days after the last NTG injection (Pradhan et al., 2014a, Guo et al., 2021). These basal responses, measured prior to NTG treatment each day, are prevented by a host of migraine preventives, including topiramate, propranolol, and amiloride, and the acute effects, measured after NTG treatment each day, aborted by sumatriptan in the chronic state (Pradhan et al., 2014a, Tipton et al., 2016, Moye et al., 2019). Thus, validating the translation of this approach. Subsequent studies have been conducted to adapt this assay, using chronic NTG (10 mg/kg) dosing to prime the trigeminal system to create a state of 'latent sensitisation'. After full recovery of mice to baseline mechanical threshold levels, around day 20, a lower dose NTG (0.1 mg/kg) is now sufficient to mediate a reduction of periorbital withdrawal thresholds (Guo et al., 2021). Latent sensitisation is not exclusive to NTG, with alternative approaches being used to prime the trigeminal system, followed by NTG or other NO donors. Examples include repeated administration of sumatriptan to mediate a proposed medication overuse headache (MOH) assay (De Felice et al., 2010b), or restraint stress (Avona et al., 2020), or inflammatory paradigms (Burgos-Vega et al., 2019). In these models, after baseline levels to mechanical stimulation are re-established, animals are now more sensitive to NO donors. While in these studies NTG was not used, the principle of using an NO donor in a primed state is potentially the same.

The mechanisms that contribute to the transition from episodic to chronic migraine in patients are largely unknown. This is somewhat complicated by an arbitrary distinction between episodic and chronic migraine (Headache Classification Committee of the International Headache Society (IHS), 2018). However, the *in vivo* NTG recurrent/chronic model of sensitisation, and adapted approaches that mediate latent sensitisation in naïve animals, offer an opportunity to gain insights into peptidergic and network-plastic changes that occur during more frequent migraine attacks, and therefore likely more representative of the recurrent nature of migraine. Indeed, insights have already been gained, with data suggesting that changes in gene expression (Jeong et al., 2018) and altered CGRP levels (Greco et al., 2018) in the trigeminal ganglia, in the chronic NTG model compared to given acutely, may contribute to the reported plastic changes in baseline sensitisation that are present in migraineurs (Torres-Ferrus et al., 2020). Further, in the MOH sensitisation paradigm using recently approved abortive drug classes, olcegepant (a gepant, CGRP receptor antagonist) treatment presented a much lower MOH-risk profile and was characterised with no changes in CGRP or Fos expression in the TNC (Saengjaroentham et al., 2020) and lack of latent sensitisation in response to both bright-light stress and a nitric oxide donor (Navratilova et al., 2020). However, for LY344864 (a ditan, 5-HT1F receptor agonist), MOH periorbital hypersensitivity developed and was accompanied by increased expression of CGRP in the TNC. These data demonstrate overlap of NTG-mediated changes with those in a MOH model, supporting the possible role of these molecular mechanisms in the transition to a more chronic migraine-like state. All studies summarized in Table 2.

Strengths of the NTG model

As a human model of migraine, NTG is an extremely reliable method to trigger a migraine-like attack in migraineurs, with up to 80 % of patients responding (Ashina et al., 2013) to NTG, which can be reproduced at a rate of over 70 % with additional NTG exposures (Akerman et al., 2019, Karsan et al., 2020). The characteristics of this migraine-like attack strongly resemble those of spontaneous migraine with overlapping pain characteristics and accompanying symptoms, to the point where they are indistinguishable **(**Table 1**)** (Ashina et al., 2013, Ashina et al., 2017, Headache Classification Committee of the International Headache Society (IHS), 2018). These migraines are accompanied by increased levels of CGRP in blood plasma samples (Juhasz et al., 2003, Juhasz et al., 2005), like spontaneous migraine (Goadsby et al., 1990, Goadsby and Edvinsson, 1993) that also similarly respond to migraine therapeutics. Furthermore, associated symptoms beyond those required for diagnosis also overlap, with 98 % (52/53) of patients reporting similar premonitory symptoms, such as tiredness, neck stiffness, as well

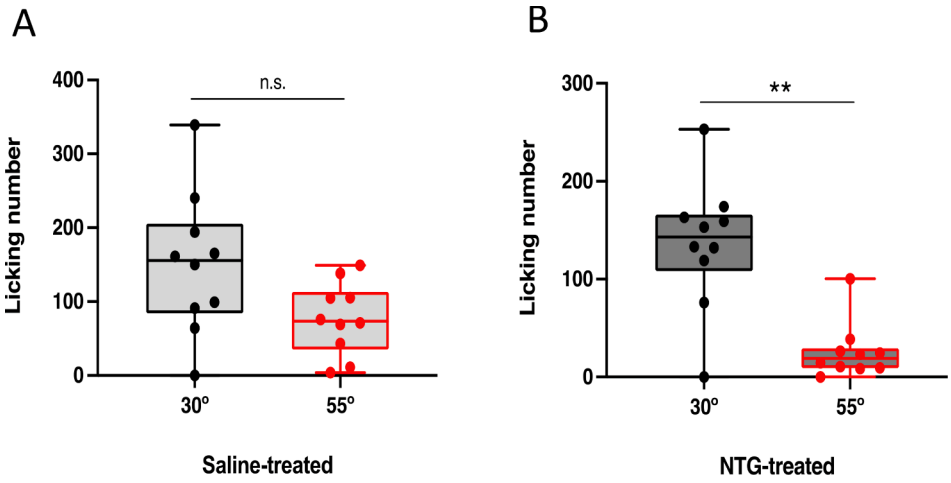

Fig. 3. Nitroglycerin (NTG) as a model of craniofacial hypersensitivity. Using the Orofacial Pain Assessment Device (OPAD), one can model the underlying sensitivity of trigeminal nociceptive pathways by applying heat within the orofacial region and measuring outcomes. Depending on the temperature of the pads surrounding a feeding device, and the underlying orofacial sensitivity, mice will choose whether to continue to lick for the rewarding condensed milk or not, which is recorded through a bottle sensor. Studies have uncovered that the reduction in the number of licks for condensed milk associated with the noxious (55°) craniofacial thermal stimulation is not significantly different from the non-noxious temperature (30°) in the **(A)** saline-treated mice. However, in **(B)** NTG-treated mice there was a significant reduction in the number of licks between the two groups, suggesting an increased orofacial thermal sensitivity. This figure is adapted and modified with permissions from (Sureda-Gibert et al., 2018).

Table 2
Summary of outcome measures in response to acute and chronic nitroglycerin (NTG) doses in rodents.

Dose	Measured outcome	Reported outcome	Therapeutic benefit	Reference
Acute NTG model				
10 mg/kg (SC) – rat	cFos immunoreactivity	↑ c-fos in trigeminal nuclei, NTS, VLM, PB, LC, amygdala, hypothalamus, PVN, supraoptic nucleus	N/A	(Tassorelli and Joseph, 1995)
2 μg/kg/min for 30 min (IV) – rat	TG electrophysiological responses	↑ Mechanosensitivity of meningeal nociceptors↑ ERK phosphorylation in meningeal arteries	Aborted by ERK blockade	(Zhang et al., 2013)
10 mg/kg (SC) – rat	TCC electrophysiological responsesPeriorbital (Von Frey) sensitivity	↑ In spontaneous and dural-evoked neural responses in the TCC↑ Sensitivity to cutaneous facial stimulation	All outcomes were reversed by a triptan, CGRP receptor antagonist, and 5-HT1F receptor agonist. NK1 receptor antagonist not effective	(Akerman et al., 2019; Akerman et al., 2021a)
10 mg/kg (IP) – rat	Anxiety-like behaviours (Elevated plus maze; EPM and open-field; OF)	↓ Time spent in open arms of EPM and entries into central zone of OF (overall ↑ anxiety-like behaviours)	Aborted by Orexin A (Ox1R agonist) in vlPAGWorsened by CB1R-antagonist vlPAG	(Pourrahimi et al., 2021)
5 and 10 mg/kg (IP) – mice	cFos immunoreactivity Hind-paw thermal (Hargreaves) and mechanical (Von Frey) sensitivityCSD (stimulus frequency, amplitude, duration and AUC)	↑ Laminae III–V of the cervical dorsal horn, TNC ↑ Hind-paw thermal hypersensitivity No changes in threshold stimulus to induce CSD	Both outcomes were alleviated by sumatriptan	Bates et al., 2009
10 mg/kg (IP) – mice	*cFos immunoreactivity* nNos immunoreactivity Periorbital (Von Frey) sensitivity	↑ *cFos in TNC*o changes in either the TNC or TG↑ Mechanical periorbital sensitivity	Reversed by topiramate but not by sumatriptan No response to topiramate or sumatriptanAborted by sumatriptan	(Farkas et al., 2016)
10 mg/kg (IP) – mice	Periorbital thermal sensitivity (Orofacial Pain Assessment Device)	↑ Thermal periorbital sensitivity		(Sureda-Gibert et al., 2018)
Chronic NTG model				
Chronic (5 doses over 9 days) 3 and 10 mg/kg – mice	Basal and chronic hind-paw sensitivity (Von Frey) Mechanical and thermal thresholds, conditioned place aversion (CPA) *peri*-orbital and hind mechanical thresholds	Development of hind-paw cutaneous allodynia at both dosesDevelopment of hind-paw mechanical and thermal hyperalgesia, and CPA to NTG (10 mg/kg) Development of periorbital and hindpaw hypersensitivity	Prevented by topiramate, acute effects reduced by sumatriptan δ-opioid agonists attenuated mechanical and thermal hyperalgesia, and abolished CPAPrevented by propranolol and amiloride, with not effect of valproate or memantine	(Pradhan et al., 2014a) (Pradhan et al., 2014b) (Tipton et al., 2016)
Chronic 10 mg/kg (IP) – mice	Basal and chronic hind-paw thermal and mechanical (Von Frey) sensitivity Periorbital mechanical (Von Frey) and cold sensitivity (acetone solution)	↑ Mechanical and thermal hyperalgesia ↑ Cold allodynia No change in periorbital mechanical allodynia	Aborted by TRP1 blocker (resiniferatoxin) Worsened by resiniferatoxin	(Kim et al., 2018)
Chronic 10 mg/kg (IP) – mice	Hind-paw and mechanical sensitivity	Development of mechanical hind-paw and periorbital hyperalgesia	Aborted by δ-opioid agonist, SNC80, given on day 10	(Moye et al., 2019)
Chronic 1–10 mg/kg (IP) – naïve and transgenic mice	Naïve miceCGRP-R and PACAP-R expression CGRPα KO micePACAP KO mice	Dose dependent ↑ in sustained periorbital mechanical allodynia↑ CGRP-R and PACAP-R neuron expression in TGNo change in eriorbital mechanical allodynia Attenuated effects of light aversion, Fos-immunoreactivity in TG and TNC	Blocked by low-dose interleukin-2 treatment	(Guo et al., 2021) (Markovics et al., 2012)
Chronic 10 mg/kg (i.p.) – mice	RNA-seq profiles of genes in the TG and NAc	Significant NTG-treatment effect over: *Per1* clock gene, *Erbb4* and *Slc1a2*, and treatment-by-region effect over: *Slc32a1* and *Penk* genes		(Jeong et al., 2018)
Chronic 5 mg/kg (i.p.) – rats	Thermal sensitivity (hot plate and tail flick tests) Spontaneous migraine-like nociceptive behaviorsAnxiety-like behaviours (EPM and OF) Photophobia (light dark box)	↑ Thermal sensitivity and tail flick responses ↑ in head shaking and freezing behaviours ↓ in time spent in open arms of EPM and entries into central zone of OF (overall ↑ anxiety-like behaviours) ↑ in time spent in dark (overall ↑ photophobia)	Not affected by orexin 1 receptor antagonist in basolateral amygdala (BLA) Both head shaking and freezing behaviours were worsened by orexin 1 receptor antagonist in BLA. Facial grooming also exacerbated All outcomes were worsened by OX1R-Antagonist (intra-BL Amygdala)	(Askari-Zahabi et al., 2022)

CB1-R, cannabinoid 1 receptor; CSD, cortical spreading depression; EPM, elevated plus maze; ERK, extracellular signal-regulated kinase; IP, intraperitoneal; IV, intravenous; LC, locus coeruleus; NAc, nucleus accumbens; nNOS, neuronal NOS; NTS, nucleus tractus solitarius; OF, open field; PAG, periaqueductal grey; PB, parabrachial; PVN, paraventricular nucleus; SC, subcutaneous; TG, trigeminal ganglion' TCC, trigeminocervical complex; TNC, trigeminal nucleus caudalis; VLM, ventral lateral medulla.

as an inability to concentrate (Afridi et al., 2004, Maniyar et al., 2014, Karsan et al., 2020), extracranial allodynia (Akerman et al., 2019), and postdrome symptoms, including vertigo and mood change, commonly present during spontaneous attacks (Karsan et al., 2021). Mapping brain activation during migraine also demonstrates a strong correlation with spontaneous and NTG-triggered migraine, based on patient imaging studies. There is overlapping evidence of brainstem (Bahra et al., 2001, Afridi et al., 2005b) and hypothalamic activation (Maniyar et al., 2014). This supports that similar mechanisms are engaged to mediate both headache outcomes.

Using NTG as a migraine trigger is also favourable compared to other exogenous triggers, such as CGRP or pituitary adenylate cyclase-activating polypeptide (PACAP) (Lassen et al., 2002, Schytz et al., 2009, Hansen et al., 2010a, Amin et al., 2014, Guo et al., 2016). Data suggest that both CGRP (approximately 60–65 %) (Guo et al., 2017b) and PACAP (approximately 65–70 %) (Guo et al., 2017c) are less reliable at triggering migraine. These migraine attacks are often also considered milder, particularly with CGRP (Lassen et al., 2002), and with a significantly lower frequency of developing premonitory symptoms, with 9 % and 48 % reporting premonitory symptoms for CGRP and PACAP, respectively (Guo et al., 2016). These data perhaps reflect the fact that NTG likely mediates the endogenous release of many transmitters involved in migraine mechanisms, including CGRP and PACAP, rather than relying on just one to mediate changes (discussed in more

detail in the 'Therapeutic Opportunities through NTG-NO models' section).

Importantly, many of these clinical features also translate to NTG preclinical models, supporting the efficacy of this approach. NTG causes activation and sensitisation of primary afferent and second-order trigeminovascular neurons (Zhang et al., 2013, Akerman et al., 2019; Akerman et al., 2021a). This is thought to be the underlying mechanism of migraine headache, and many craniofacial nociceptive symptoms. NTG also mediates facial cutaneous and extracephalic (hind-paw) hypersensitivity to somatosensory probing in mice (Bates et al., 2009, Pradhan et al., 2014a, Moye et al., 2019, Holton et al., 2020), indicative of allodynia and the behavioral correlate of neuronal sensitisation. Both these physiological and behavioral outcomes respond to established migraine therapeutics, also demonstrated in patients (Akerman et al., 2019).

Fos/*c-fos* expression studies have allowed the mapping of brain regions activated in response to NTG in rodents (Tassorelli and Joseph, 1995, Tassorelli et al., 1999). These studies demonstrate strong overlap with migraine-associated nuclei that are also responsive to durovascular stimulation, including; the spinal trigeminal nucleus and upper cervical spinal cord (Kaube et al., 1992, Strassman et al., 1993, Strassman et al., 1994), periaqueductal gray (PAG) (Hoskin et al., 2001), hypothalamic nuclei including paraventricular and posterior (Benjamin et al., 2004, Robert et al., 2013), parabrachial nucleus (Malick et al., 2001, Ter Horst et al., 2001), and thalamic nuclei (Park et al., 2014), and have been instrumental in advancing our understanding of the trigeminovascular system. NTG-induced *c-fos* immunoreactivity has also identified other networks/hubs such as the LC/dorsal rostral pons and the hypothalamus (Tassorelli and Joseph, 1995), which clinically also appear to be functionally altered prior, during, and even after migraine pain (Afridi et al., 2005a, Moulton et al., 2008, Maniyar et al., 2014, Schulte and May, 2016, Schulte et al., 2020).

Despite the challenges in accurately and robustly measuring complex behavioural responses in animal models recent work has also reliably reported migraine-like associated phenotypes such as fatigue, photophobia or appetite and altered sleep patterns, in the form of reduced locomotion, photophobia (Sufka et al., 2016), reduced cognitive performance and increased anxiety-related behaviours (Taheri et al., 2020). All of which, again, are similarly reported in both spontaneous and NTG-triggered attacks.

Having observed similar patterns between post-hoc measurements and patient functional imaging studies, one could argue that there is an even larger translational gap between *in vitro* region-localised observations versus *in vivo* network-specific findings, when preclinical approaches are compared to clinical observations. Recent advances however aim to strengthen the translatability of the NTG model by establishing similar functional imaging paradigms in animal models (Harrington et al., 2011), with one study demonstrating promising functional connectivity patterns between the PAG and migraine-associated structures in a model of duro-vascular-stimulation (Jia et al., 2017).

Weaknesses of the NTG-model

While the NTG model of migraine, both in humans and animals, has considerable strengths, it is not without its weaknesses. Firstly, not every patient responds to NTG with a migraine-like attack. This likely reflects patient heterogeneity, something that can also be argued with triggers of spontaneous migraine, and that NO is likely only one of many neurotransmitters involved in migraine mechanisms. This is also borne out more generally in the variety of treatments that are effective for migraine that target different transmitter and receptor systems, and that not all patients are responders to all treatments. Similarly, while NTG-triggered migraine is responsive to sumatriptan, aspirin, and valproate (Tvedskov et al., 2004a, Juhasz et al., 2005, Akerman et al., 2019) these migraines were less responsive to olcegepant and propranolol (Tvedskov et al., 2004b, Tvedskov et al., 2010). Although, these may reflect less than optimal study design with respect to dosing regimen. There is also conflicting data with respect to cerebrovascular changes. In one study NTG did not mediate significant vasodilation of cerebral or meningeal arteries (Schoonman et al., 2008), whereas in spontaneous migraine evidence has been demonstrated of significant changes (Friberg et al., 1991). Again, this may reflect different mechanisms involved but may also support the general belief that cerebrovascular changes are an epiphenomenon in migraine, a response to trigeminovascular activation that is not necessary for migraine-related headache.

When it comes to preclinical translation, a common concern lies in the reproducibility of NTG models from the bench to the bedside. Naïve rodents require significantly higher doses to mediate activation and sensitisation of trigeminovascular neurons compared to migraineurs and certain genetic models. Studies have ranged from the equivalent of 60 μg/kg (IV) to 5–10 mg/kg (IP or SC) in mice and rats. This covers a range or 6–1000 times higher than the human dose, and an understandably concerning gap between species. That said, lower dosing (such as 1 mg/kg, SC) has consistently not returned significant changes (Bates et al., 2009, Akerman et al., 2019). Great effort has therefore focused on applying 'Body Surface Area' (BSA) formulas to facilitate drug conversions from animals to humans, by considering the common height/weight ratio in both species (Reagan-Shaw et al., 2008). Based on this, NTG dose conversion might be smaller than often reported: with a 5 mg/kg dose in mice being equivalent to 0.4 mg/kg in humans (i.e. 11.5 times difference). In addition; given the variability in drug absorbance and clearance among species depending on the route of administration (Thomsen and Olesen, 1997, Christiansen et al., 2000, Sances et al., 2004), conversion paradigms would also benefit from considering the mechanisms of elimination of a drug. Further, the liver's vascular endothelial cell structure, which is responsible for converting NTG into NO, has been reported to be significantly different in mice: having higher rates of endothelial cell proliferation, compared to humans and rats. In addition, these are naïve rodents rather than 'migrainous'. Together, these data go some way to explain the disparity between human dosing and that required to mediate a response in rodents. Despite these limitations, this NTG dosing approach is validated by its reliable response to established and newly approved drug classes, including triptans, NSAIDs such as aspirin, gepants, ditans, and migraine preventives (Pardutz et al., 2004, Bates et al., 2009, Pradhan et al., 2014a, Akerman et al., 2019, Akerman et al., 2021a), and it is being utilised to dissect novel targets, discussed in the next section. Even though there is strong translation of the rodent NTG model, it is now also being adapted and evolved so that more human-like doses of NTG are effective at mediating migraine-like responses. One approach has been to mediate a 'latent sensitisation' in rodents, where prior chronic dosing with NTG (Guo et al., 2021), triptan overuse (De Felice et al., 2010a,b), or even restraint stress (Avona et al., 2020, Kopruszinski et al., 2021) unmask a more prolonged sensitivity to NO donors, such as NTG, so lower doses are now able to trigger hypersensitive responses. Another approach is to use the subpopulation of Sprague Dawley rats that demonstrate a 'spontaneous trigeminal allodynia' (Oshinsky et al., 2012). These rats appear to have inherited migraine-like traits, including demonstrating hypersensitive craniofacial responses to lower NTG doses (0.1 mg/kg, IP).

Comparisons between NTG preclinical models also remains a challenge due to inter-species variability. While some studies report limited periorbital mechanical hypersensitivity to an acute dose of NTG (10 mg/kg) in rats (Sufka et al., 2016), others observe increased tail-flick (Greco et al., 2008) and orofacial responses to formalin (Demartini et al., 2017) with the same dosing. Other behavioural models measuring thermal sensitivity have also reported increased sensitivity post-NTG (10 mg/kg) in mice (Sureda-Gibert et al., 2018) but not in rats (Caudle et al., 2020). It also remains unclear whether these differences can be attributed to the type of NTG formulation (Farkas *et al.,* 2015) and/or underlying biological parameters, such as sex (Boes and Levy, 2012, Greco et al., 2013),

stress (Raoof et al., 2022), or the microbiome composition (Lanza et al., 2021, Kang et al., 2022), all of which can contribute to NTG-induced hyperalgesia in rodents in a different manner.

Another commonly reported weakness of the NTG model in humans is its inability to trigger migraine with aura (Christiansen et al., 1999, Sances et al., 2004), with only around 14 % of patients developing aura post-NTG. Similarly, patients with either familial hemiplegic migraine 1 or 2 (FHM1 or 2) are far less sensitive to NTG compared to those with only migraine with or without aura (Hansen et al., 2008, Hansen et al., 2010b). While significant progress in the development of preclinical models of aura has heralded therapeutic successes and our mechanistic understanding supports that perhaps migraine without aura may involve different mechanisms compared to migraine with aura (Diener et al., 2015), NTG models are still unable to address and dissect the underlying pathophysiological mechanisms of the 30 % of migraineurs that suffer aura symptoms.

Therapeutics opportunities through NTG-NO models

As well as phenotypic overlap with migraine, NTG-NO migraine models also offer unique opportunities for drug discovery, either as a screen to predict potential therapeutic efficacy or by targeting the molecular pathways thought to be activated by NTG in triggering migraine (Demartini et al., 2019). Here, we highlight examples that validate the NTG model using new approved therapeutics, as well as more novel opportunities that have future potential as therapeutic targets.

NTG mediates the release of various neuropeptides, including CGRP and PACAP, and each is released into the cranial vasculature during migraine (Goadsby et al., 1990, Zagami et al., 2014). Like NTG, both also trigger migraine in migraineurs, although they are considered less effective migraine triggers (Lassen et al., 2002, Schytz et al., 2009, Hansen et al., 2010a, Amin et al., 2014, Guo et al., 2016) and each mediates activation and sensitisation of trigeminovascular neurons (Akerman and Goadsby, 2015, Akerman et al., 2019, Akerman and Romero-Reyes, 2020, Akerman et al., 2021a) when given exogenously. In migraine patients NTG-induced migraine is associated with an increase in plasma CGRP that is normalized with triptan treatment resulting in migraine relief (Juhasz et al., 2003, Juhasz et al., 2005). In rodent models NTG increases CGRP levels in plasma (Di et al., 2015) and promotes CGRP release in the TNC (Pardutz et al., 2002, Demartini et al., 2019), while CGRP knockout mice do not respond to NTG with migraine-like behaviors (Guo et al., 2021). NTG also elevates immunoreactivity of PACAP-27 and −38 in the TNC of rats (Tuka et al., 2012) and NTG-induced trigeminal neuronal activity and meningeal vasodilation are significantly attenuated in PACAP$^{-/-}$ knock out mice (Markovics et al., 2012). Repeated NTG administration in mice upregulates the number of both CGRP and PACAP receptors in trigeminal ganglion cells, whereas a single NTG dose has little effect on either (Guo et al., 2021).

This builds the picture that NTG-mediated neurotransmitter mechanisms through CGRP and PACAP have considerable overlap with mechanisms in spontaneous migraine. Importantly, molecules targeting the CGRP pathway are now approved and effective in the treatment and prevention of migraine (Hargreaves and Olesen, 2019). Similarly, CGRP receptor antagonists are effective in reducing NTG-mediated migraine-like response in rodents (Greco et al., 2014, Akerman et al., 2021a); supporting the translation of this model. Further, several preclinical studies also support targeting PACAP receptors in the treatment of migraine (Boni et al., 2009, Akerman and Goadsby, 2015, Rubio-Beltran et al., 2018, Hoffmann et al., 2020), with PACAP targeted therapies currently in development and being trialed (Moreno-Ajona et al., 2021). Together, data using NTG in clinical and preclinical studies would imply a significant role of CGRP and PACAP and would predict that targeting these peptides would be predictive of therapeutic efficacy. This has of course been confirmed with CGRP, with studies still ongoing for PACAP. But, this lends strong support for the translation of the NTG model and its ability to dissect and predict potential novel targets. Adding further support for this, a 5-HT1F receptor agonist, from the new ditan class of migraine abortives, is also able to abort NTG-mediated activation and sensitisation of trigeminovascular neurons in a rodent model (Akerman et al., 2021a).

Despite much positive data related to NTG and CGRP-target therapies olcegepant was not able to prevent NTG-triggered migraine in patients (Tvedskov et al., 2010). This is a curious dataset that suggests that CGRP might not be as important in NTG-triggered migraine than previously believed. However, study design and the timing of olcegepant administration may have restricted its potential efficacy. Here, it was given as a preventive after NTG infusion before any symptoms of migraine-like headache developed. Peak headache severity was reported between 250 and 400 min post NTG. However, olcegepant has a half-life of approximately 150 min (Iovino et al., 2004), long before headache severity peaked. In previous studies NTG-triggered migraine is successfully aborted or attenuated by triptans, administered after migraine headache has developed (Iversen and Olesen, 1996, Afridi et al., 2004). It is very possible that the lack of efficacy in this study might reflect more the sub-optimal time of administration than an actual lack of a role of CGRP in NTG-mediated effects. It is also an important illustration of the importance of timing in treatment regimens. While treating early in migraine is considered crucial to therapeutic success (Goadsby et al., 2008), treating too early, before even migraine symptoms begin, using a drug with a relatively short half-life, may be equally relevant. Also, given that NTG mediates the release of many neurotransmitters, including CGRP, it is plausible to speculate different underlying pathophysiological mechanisms among patients, with subtypes of patients that have either CGRP-dependent or CGRP-independent migraine attacks (Alpuente et al., 2022), or where CGRP is one of several relevant transmitters.

Beyond CGRP and PACAP, using NTG has also opened up opportunities for novel discovery efforts. Activation of the glutamatergic pathway is believed to be involved in central sensitization in migraine (Hoffmann and Charles, 2018, Demartini et al., 2019) and increased glutamate levels in the CSF of chronic migraine patients is associated with higher pain scores (Peres et al., 2004). Furthermore, memantine has demonstrated efficacy in the prevention of migraine (Charles et al., 2007). While in animal models, NTG increases plasma glutamate levels in rats (Gao et al., 2014) and blocking the kynurenic acid pathway is anti-nociceptive in the NTG model (Greco et al., 2017). There is also engagement of the endocannabinoid system by NTG and NO donors. NTG and NO donor-mediated trigeminovascular changes are attenuated by anandamide (Akerman et al., 2004, Greco et al., 2011). NTG administration also increases the activity of fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL) in midbrain, medulla, and hypothalamic regions, hydrolysing enzymes that promote the breakdown of endogenous endocannabinoids (Greco et al., 2010). Inhibiting MAGL and FAAH significantly attenuates nociceptive behavioral outcomes in the NTG model (Greco et al., 2015, Greco et al., 2020, Greco et al., 2021a, Greco et al., 2021b).

Inflammatory mechanisms and the immune system have also been implicated from NTG studies. Brain-derived neurotrophic factor (BDNF) is up-regulated in the trigeminal ganglion of chronic NTG-treated rats (Guo et al., 2017a). Increased serum levels of BDNF were found in the same animals, which parallels findings in patients during migraine (Fischer et al., 2012). NTG also activates inflammatory pathways, causing upregulation of pro-inflammatory mediators including interleukin 1β (IL-1β) and IL-6 in dura mater and dural macrophages, respectively (Reuter et al., 2001). There is also increase in nuclear factor kappa B (NF-κB) and iNOS expression (Reuter et al., 2002). Together, these lead to an inflammatory cascade that includes dural plasma protein extravasation and mast cell degranulation as part of a potential migraine-like mechanism (Reuter et al., 2001, Reuter et al., 2002).

Beyond glutamatergic and inflammatory mechanisms are other potentially novel receptor targets that have been dissected as a

consequence of preclinical NTG-related studies. Delta-opioid receptor agonists inhibit both mechanical and thermal hind-paw hyperalgesia and periorbital hypersensitivity, as well as NTG-induced conditioned place aversion, in the chronic NTG migraine model (Pradhan et al., 2014b, Moye et al., 2019). These data are strengthened by the fact that delta-opioid receptor agonists are also effective in a model of cortical spreading depression (Pradhan et al., 2014b) and in models of MOH and post-traumatic headache (Moye et al., 2019). Further data support the engagement of the delta-opioid receptor in the therapeutic action of vagus nerve stimulation in the treatment of migraine (Hu et al., 2021). Histone deacetylases (HDACs) are similarly implicated in migraine mechanisms. HDAC6 specifically is involved in deacetylation of microtubules making them more fragile and prone to breakage (Bertels et al., 2021). This impacts response to nerve injury, neuronal signaling, and axonal transport, with implications in disease chronicity potentially related to pain. Inhibiting HDAC6 may therefore have therapeutic potential (Bertels et al., 2021). Indeed, chronic NTG was found to decrease neurite growth in the TNC, PAG, and somatosensory cortex and treatment with an HDAC6 inhibitor restores neuronal complexity and reverses NTG-mediated periorbital hypersensitivity. These studies provide potential new opportunities for drug discovery in migraine as well as understanding migraine mechanisms. With this, future studies are required to ultimately determine the potential of these novel findings.

Finally, and perhaps sitting somewhere between strengths and weaknesses of NTG as a migraine model, is the progress in the development of therapeutics targeting NO, borne out of NTG and NO-mechanistic studies in both humans and animals. NTG is thought to mediate its mechanism in triggering migraine-like symptoms as an NO donor. While the exact role of NO and its mechanism in spontaneous migraine is not known, the importance of NO is supported by a clinical trial demonstrating that NOS inhibition, using N(G)-monomethyl-L-arginine (L-NMMA), a pan-NOS inhibitor (NOSi), was effective at treating spontaneous migraine attacks (Lassen et al., 1998). These data are further supported by preclinical studies using NOSi (Messlinger et al., 2000, Akerman et al., 2002, De Col et al., 2003, Akerman et al., 2021b). Specfically, these data support the hypothesis that nitrergic mechanisms play a significant role in migraine, and that potentially selective NOS inhibition would be efficacious and a potential novel target in the treatment of migraine. Initial work focused on inducible (i) NOS as a target. INOS is increased in dural meningeal macrophage after NTG administration in rats (Reuter et al., 2001), with the subsequent inflammatory cascade blocked using an iNOS inhibitor (Reuter et al., 2001, Reuter et al., 2002). Similarly, studies in patients have also revealed upregulated iNOS in blood samples taken during spontaneous migraine (Sarchielli et al., 2006). However, GW274150, a potent and selective iNOS inhibitor, failed in clinical trial as both an abortive (Palmer et al., 2009) and preventive (Hoivik et al., 2010) treatment in migraine. This is supported by data using a preclinical model of acute nociceptive dural-trigeminovascular activation, with no inflammatory component (Akerman et al., 2002, De Col et al., 2003). Targeting neuronal (n)NOS has produced inconclusive results (Messlinger et al., 2000, Akerman et al., 2002, Lambert et al., 2004, De Felice et al., 2010a, Bhatt et al., 2013), with some studies showing efficacy in preclinical models while others fail to produce a signal. With no clinical trials completed/reported for nNOSi in migraine patients it is still to be proven whether the translation of using NTG for migraine can produce a useful target in the treatment of migraine based on a specific nitrergic mechanism.

However, other nitrergic opportunities may exist. NO belongs to a class of molecules known as reactive nitroxidative species that amongst their properties include oxidative, nitrosative, and nitroxidative stress. Similar molecules include superoxide (O_2^-; SO) and peroxynitrite ($ONOO^-$; PN), which is the direct reaction product of NO and SO (Beckman et al., 1990, Little et al., 2012b), and therefore may also represent an NO-related target. Indeed, studies over the last decade have demonstrated the critical role of PN in the development and maintenance of spinal sensitization associated with persistent neuropathic and inflammatory pain (Muscoli et al., 2007, Salvemini et al., 2011, Doyle et al., 2012, Little et al., 2012a, Janes et al., 2013). Further, targeting PN production both prevents and aborts nociceptive responses, particularly in chronic models, providing compelling evidence as a novel target for pain management (Salvemini et al., 2011, Little et al., 2012a, b). PN has also been linked to migraine mechanisms with one study demonstrating that using blood platelet samples, in the presence of the NO precursor, L-arginine, PN production was significantly higher in the samples of headache-free migraine patients compared to healthy controls (Taffi et al., 2005). This suggests that migraine patients are more responsive to NO precursor molecules that mediate PN production, even outside of an attack. In a preclinical study a PN decomposition catalyst was equally effective as a NOSi at inhibiting migraine-like dural trigeminovascular responses. However, it was significantly more effective than the NOSi at inhibiting CGRP-induced activation and sensitisation of dural-trigeminovascular neurons and preventing periorbital hypersensitivity (Akerman et al., 2021b). Clearly, further studies using NTG, and other migraine models are necessary to validate PN, a direct product of NO production, as a novel target involved in mechanisms of migraine. But these data suggest that modulating NO or other reactive nitroxidative species, either directly or indirectly, remains a potential pathway for the development of novel migraine therapies and therefore involved in migraine mechanisms.

Future research perspectives/conclusions

As evidenced, the use of NTG in clinical research provides a reliable model of migraine given the strong resemblance to spontaneous migraine attacks (Goadsby et al., 1990, Fullerton et al., 1999) that carries significant benefits over other provocative agents. It is however also clear that while it can predict the efficacy of some therapeutic targets in animals (Akerman et al., 2019, Akerman et al., 2021a), there are still limitations with respect to human studies (Tvedskov et al., 2004a, Tvedskov et al., 2004b, Tvedskov et al., 2010). Similarly, NTG is a less effective model for investigating other migraine-associated symptoms, such as aura (Christiansen et al., 1999, Sances et al., 2004). However, preclinical research has strongly benefited from the translation of this approach in animals, predicting the efficacy of established and recently approved therapeutics (Bates et al., 2009, Pradhan et al., 2014a, Tipton et al., 2016, Akerman et al., 2019, Akerman et al., 2021a) and in dissecting the role of specific pathways, including a host of novel mechanisms with potential as targets, including glutamate, BDNF, pro-inflammatory mechanisms, delta-opioid receptors, HDAC6, and reactive nitroxidative species. Again, there is caution as the outcome measures have not always been reproducible across rodent models and research centers, these represent new opportunities borne out of NTG-NO studies.

While NTG does not uniformly trigger migraine-like attacks in all patients, these discrepancies may reflect different subtypes of migrainous brains; which might lead to distinct responses to triggers, as well as treatments (Alpuente et al., 2022). Indeed, genetic preclinical models are a great example of a different underlying susceptibility, which makes them more likely to express allodynic phenotypes at lower NTG doses (Brennan et al., 2013), compared to naïve animals. With this, and the use of advanced genetic modelling techniques, which allow gene editing in rodents (Harriott et al., 2019), there is broad scope to continue to dissect NO-dependent mechanisms in models of genetic susceptibility. It is noteworthy that NTG is capable of inducing similar migraine-associated features, including migraine pain/nociception and sensory disturbances, in both patients and animal models, suggesting a central and overlapping mechanism of action. Indeed, recent efforts have dissected potential peptides and/or molecules which might be involved in generating other migraine-associated phenotypes such as anxiety (Askari-Zahabi et al., 2022), photophobia (Casili et al., 2020) or locomotor deficits (Pourrahimi et al., 2021). The use of NTG models, in

combination with novel viral-based technologies, which allow *in vivo* and controlled manipulation of specific neural types (Houben et al., 2017), are an exciting approach to further unravel the underlying mechanisms of NTG-induced sensitization.

Lastly, it is often a challenge to translate all the relevant clinical information, whether that is imaging or symptomatology, to the bench due to the complexity of networks involved but also the heterogeneity of the disorder, between and even within each patient. No animal model is perfect, each with its limitations alongside their benefits. Despite this, clinical NTG models seem to be able to establish a 'common ground' or 'common mechanism of action' among a majority of migraineurs, which does translate to a significant degree, and can be used to further dissect mechanisms (both pathophysiological and treatment) in associated preclinical models. With recent developments in genetic, imaging, histological and viral-based technologies, using NTG still offers a great scope of opportunities to dissect the downstream pathophysiological mechanisms of migraine and uncover potential novel targets for discovery.

CRediT authorship contribution statement

Paula Sureda-Gibert: Conceptualization, Writing – original draft, Writing – review & editing, Visualization. **Marcela Romero-Reyes:** Writing – original draft, Writing – review & editing. **Simon Akerman:** Conceptualization, Writing – original draft, Writing – review & editing, Supervision, Visualization.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

MRR – reports personal fees and grant funding from Amgen, and personal fees from Allergan, Kallyope, and Patent/Legal work in headache and orofacial pain, unrelated to this work.

SA – reports personal fees and grant funding from Amgen, and personal fees from Allergan, Kallyope, and Patent/Legal work in headache and orofacial pain, unrelated to this work.

The remaining author declares that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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