

REVIEW ARTICLE

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Animal models of migraine and experimental techniques used to examine trigeminal sensory processing

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Abstract

Background: Migraine is a common debilitating condition whose main attributes are severe recurrent headaches with accompanying sensitivity to light and sound, nausea and vomiting. Migraine-related pain is a major cause of its accompanying disability and can encumber almost every aspect of daily life.

Main body: Advancements in our understanding of the neurobiology of migraine headache have come in large from basic science research utilizing small animal models of migraine-related pain. In this current review, we aim to describe several commonly utilized preclinical models of migraine. We will discuss the diverse array of methodologies for triggering and measuring migraine-related pain phenotypes and highlight briefly specific advantages and limitations therein. Finally, we will address potential future challenges/opportunities to refine existing and develop novel preclinical models of migraine that move beyond migraine-related pain and expand into alternate migraine-related phenotypes.

Conclusion: Several well validated animal models of pain relevant for headache exist, the researcher should consider the advantages and limitations of each model before selecting the most appropriate to answer the specific research question. Further, we should continually strive to refine existing and generate new animal and non-animal models that have the ability to advance our understanding of head pain as well as non-pain symptoms of primary headache disorders.

Keywords: Pain, Migraine, Headache, Preclinical, Animal models, Electrophysiology, Translation

Background

Migraine is a debilitating condition whose main attributes are severe recurrent headaches with accompanying sensitivity to light and sound, nausea and vomiting. It is a highly prevalent and heterogeneous neurological disorder affecting approximately 6% of men and 18% of women [1] and is mediated by a combination of genetic [2] and environmental factors [3]. The pain associated with migraine is a major cause of its accompanying disability and can encumber almost every aspect of daily living [4, 5]. The disability associated with migraine underscores the need for selective and effective therapeutic tools. To that end, advancements in the neurobiology of migraine headache have come, in large

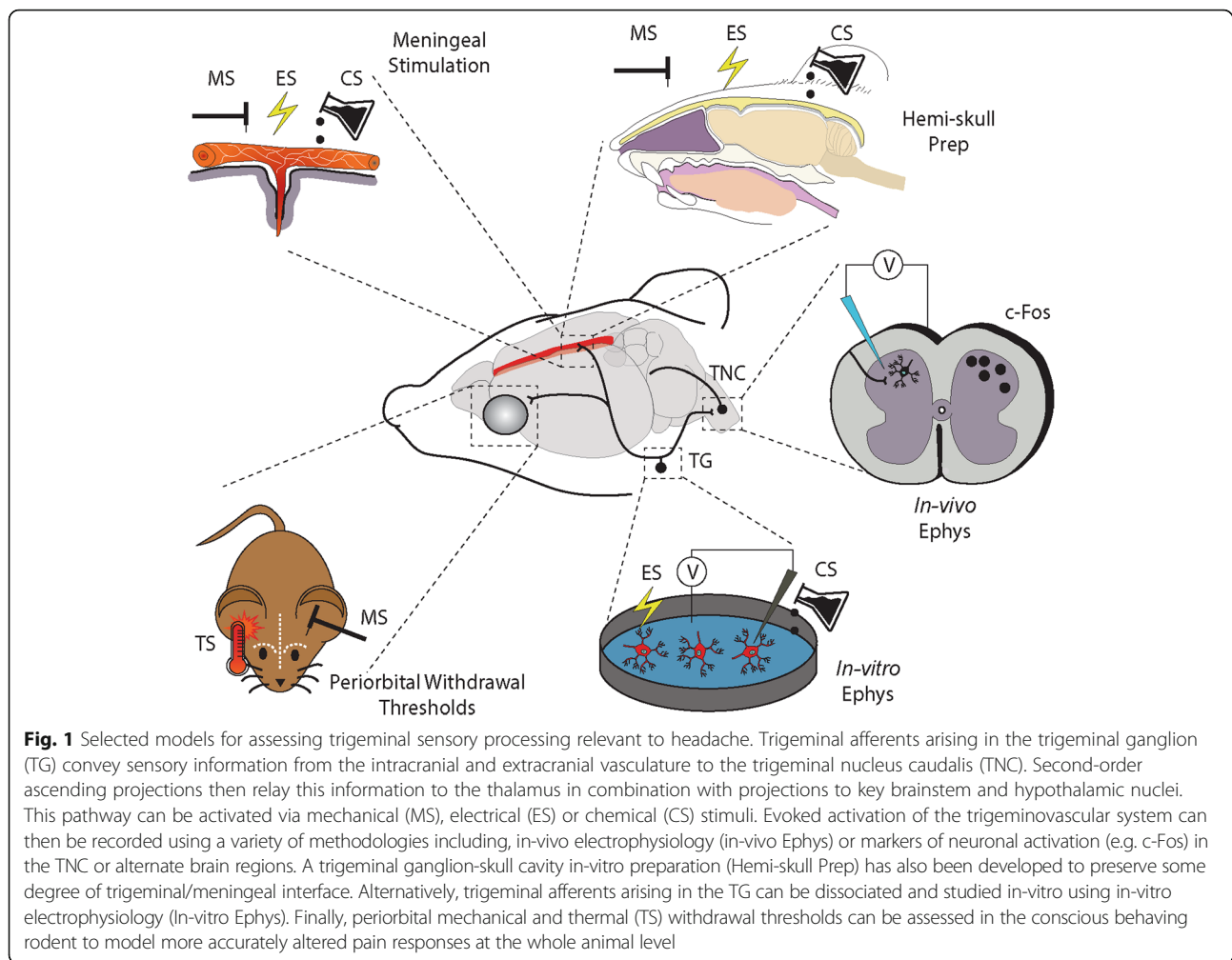
part, from basic science research utilizing small animal models of migraine-related pain [6, 7] (Fig. 1). The recent development of new antibody drugs to treat migraine pain [8, 9] and pipeline therapies in development [10] is a testament to the translational potential of the animal models of migraine. Despite this, the complexity of migraine has been an impediment to fully modeling the disorder in animals and remains a major hurdle to overcome.

Several lines of evidence suggest that activation of trigeminal nociceptors innervating meningeal tissues including dural arteries and sinuses is central to the initiation of migraine pain [11]. Stimulation of these trigeminovascular afferents in humans can reproduce referred pain with qualitative similarity to migraine in cephalic regions [12]. While the central mechanisms by which trigeminovascular afferents are activated remains ill defined, once activated, they likely release neuropeptides including calcitonin gene related peptide (CGRP),

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substance P, neurokinin A and pituitary adenylate cyclase activating peptide (PACAP) in the dura, and centrally in the brainstem [13–15]. Release of CGRP peripherally from nociceptive afferents may mediate additional release of mast cell contents and other immune mediators [16]. Subsequent sensitization of trigeminovascular afferents and central sensitization of second order neurons in the trigeminal nucleus caudalis [17] are important component parts of migraine neurobiology that animal models ultimately seek to reproduce [11]. In this review, we aim to describe the current array of preclinical models available to researchers in the field. The diverse array of methodologies for triggering and measuring migraine-related pain phenotypes will be discussed and we will briefly highlight potential novel technologies and genetic tools that we feel will have a significant impact on preclinical migraine research in the next decade. We also highlight some clinical correlates to the models being studied, their emergence from clinical observation, potential in therapeutic testing and the extrapolation of their findings to the human experience of migraine.

Animal models of migraine focused on trigeminal sensory processing have increased our mechanistic understanding of migraine pathobiology and have direct implications for target identification and translational research. Data from the models described has led to advances in drug therapy and a better comprehension of the mechanisms of drugs and devices currently approved for the treatment of migraine. Lastly, use of these models increased our understanding of the complex interplay of sex, environment and genetic influences on pain processing and migraine pathobiology. In addition to the following discussion, the reader is directed to several highly relevant review articles that complement the content herein [7, 18–22].

Modeling migraine pain in the animal - experimental approaches

In-vitro application of inflammatory mediators to dissociated meningeal afferents

Release of algogenic and inflammatory substances including nitric oxide, CGRP, neurokinin A, substance P,

prostaglandins, and cytokines in the meninges are thought to influence the activation of trigeminovascular afferents [13, 23–25]. Moreover, in addition to the abundance of neuropeptide containing afferent terminals [26–29] meningeal tissues have a rich supply of mast cells. Mast cell degranulation may increase meningeal concentrations of histamine, serotonin and bradykinin further impacting trigeminovascular afferents [30–32].

The mechanisms of this trigeminovascular afferent activation can be studied in-vitro using application of these inflammatory substances to acutely dissociated or cultured trigeminal neurons. This includes targeted approaches using retrograde tracers from the dura mater to ensure the selection of acutely dissociated meningeal afferents. Electrophysiology techniques can then be utilized to examine changes specific for meningeal afferent excitability [33], ion channel modulation [34, 35] and afferent responses to current or novel migraine therapeutic targets [34], in the presence and absence of inflammatory mediators. For example, results from this model support inflammatory mediator-induced sensitization of trigeminovascular afferent nerve terminals via increases in tetrodotoxin resistant sodium currents, decreases in calcium dependent potassium currents, activation of a calcium dependent chloride current and increases in intracellular calcium signaling [35]. Furthermore, data from this model provided evidence that sumatriptan, a 5-HT_{1B/1D} receptor agonist and migraine abortive drug, can inhibit voltage-gated calcium currents and produce hyperpolarizing shifts in voltage-gated potassium currents in meningeal afferents [34]. Importantly, given the gender bias in migraine, specific sexually dimorphic responses have also been noted. Specifically, there was a greater proportion of dural afferents sensitized by inflammatory mediators in female as compared to male rats which may reflect sex-differences in activation of intracellular second-messenger pathways. There were also sex differences in active electrophysiological properties of the action potential waveform in females as compared to males following inflammatory mediator exposure suggesting differential inflammation induced modulation of voltage gated ion channels [36]. Additionally, in-vitro trigeminal neuron cultures can be combined with genetically modified animals (see Future Perspectives section) to examine changes more specific to migraine pathobiology. For example, trigeminal ganglion neurons isolated from mice expressing genetic mutations responsible for familial hemiplegic migraine (FHM) type 1 (R192Q mutation of the *CACNA1A* gene) were used to examine calcium/calmodulin dependent protein kinase II mediated increases in purinergic signaling [37].

Despite these important observations and the high-throughput nature of this model, there are several limitations that need to be carefully considered. The acute dissociation of ganglion neurons augments the distribution of proteins in the membrane and may cause some nerve injury and alterations in neuronal excitability, with important implications for migraine biology [38]. Additionally, the isolated nature of this model that can provide excellent mechanistic focus on meningeal afferents, also precludes the ability to study the interaction with other neuronal and non-neuronal populations including sympathetic and parasympathetic neurons and ganglionic satellite glial cells which may obfuscate extrapolation of the data obtained with this in-vitro model to the in-vivo environment. While such limitations are somewhat addressed in the adapted trigeminal ganglion-skull cavity in-vitro preparation (hemi-skull preparation) that attempts to preserve the hemi-dura, studies of meningeal inflammation are difficult. Nonetheless, using the in-vitro hemi-dura preparation, investigators were able to show that electrical stimulation of the trigeminal ganglion and application of inflammatory mediators on the dura increased meningeal CGRP release and produced longer-lasting increases in prostaglandin E2 [39]. Furthermore, in-vitro techniques are not commonly used to examine chronic or repetitive application of inflammatory substances over time. Therefore, the recurrent nature of migraine cannot be studied using this model. The observed findings, however, form an important basis for the determination of potential novel mechanisms in migraine-related pain and the in-vitro nature of the model has important ethical advantages, allowing relatively high throughput screening combined with a potential reduction in animal use [40].

Direct electrical stimulation of trigeminal neurons in-vivo

There are currently three principal migraine models used to directly stimulate trigeminal neurons in-vivo. These models have been improved overtime to lessen their invasiveness and allow for chronic experimentation [18]. The first involves electrical stimulation of the trigeminal ganglion, the second electrical stimulation of the meningeal nerve terminal and the third chemical stimulation of meningeal afferent nerve terminals (see Administration of inflammatory substances to the meninges in-vivo section).

Firstly, the trigeminal ganglion of anesthetized animals can be electrically stimulated using inserted stereotactic bipolar electrodes. Trigeminal ganglion neurons are then activated using low frequency (~ 5-Hz) stimulation [41–43]. The benefit of this model is that tissue specific changes in the meninges and activation of central neurons and their response to drug therapy can be examined more directly as compared to in-vitro models. For example, data from this

model has demonstrated that trigeminal ganglion stimulation causes release of CGRP from perivascular afferent terminals innervating the meninges. This release was accompanied by triptan sensitive ultrastructural morphometric changes in the neuropeptide containing nerve terminal swellings [41, 42]. While trigeminal ganglion electrical stimulation also produces activation of neurons in the trigeminal nucleus caudalis (as measured by expression of immediate early genes; see Immunohistochemistry section), this activation was not modulated by administration of sumatriptan [42]. While certain studies utilized prolonged stimulation paradigms (approximately 30 min) that may be considered supramaximal to induce morphological changes, shorter ganglionic stimulation (3–5 min) protocols also elicit peripheral neuropeptide release that is responsive to the triptans and dihydroergotamine [44]. The ability to target the trigeminal ganglion directly has many advantages, given its key role in the pathophysiology of migraine-related pain. Importantly, evidence using this approach support triptan and dihydroergotamine induced inhibition of peripheral neuropeptide release as a plausible mechanism of anti-nociceptive action [44]. However, the need to insert stimulating electrodes deep into the brain parenchyma has the potential to generate inflammatory responses both locally on the dura mater and throughout the central nervous regions traversed by the electrodes.

Secondly, similar to stimulation of the trigeminal ganglion, electrical stimulation of meningeal nerve terminals innervating the superior sagittal sinus [15], transverse sinus [45] or middle meningeal arteries [46, 47] to elicit trigeminal afferent activation have also been used to model migraine preclinically. These approaches evolved from the demonstration in humans: that their stimulation was considered painful and that this pain was often referred to the face [12] and that their stimulation in cats produced similar alterations in neuropeptide release to migraine patients [13, 15]. Moreover, direct stimulation of the intracranial vessels and subsequent activation of the meningeal afferents that innervate them, leads to the polysynaptic activation of the central projection sites of these afferents in the trigeminal nucleus caudalis and ascending projections throughout the central nervous system [48–51]. Such studies have proven critical in the ability to identify specific migraine-related pain processing nuclei throughout the brain. Furthermore, direct stimulation of nerve terminals innervating the intracranial vasculature and their meningeal afferents has proven a robust model to test differential responses to drug administration [45–47, 52, 53], similar to direct stimulation of the trigeminal ganglion as mentioned above [54, 55]. Importantly, this pharmacological testing has proven to be highly predictive of translational efficacy, both in terms of positive translation [46, 56, 57], therapeutic potential [58] and clinical trial failure [59], highlighting

their continued utility. While in-vivo electrical stimulation models better account for the biological complexity of disease as compared to in-vitro models, they are limited by the invasiveness of craniotomy and tissue exposure. Additionally, upstream events leading to trigeminal activation are bypassed and stimulation parameters must be carefully regulated to prevent supramaximal stimulation that may not adequately represent a physiological state [18]. Animals are anesthetized and therefore different anesthetic regimes need to be considered. Lastly, while these models represent surrogate readouts of trigeminal nociceptive activation at the specific recording site, they do not incorporate many aspects of pain, or determine the overall pain phenotype at the whole animal level.

Administration of inflammatory substances to the meninges in-vivo

Several experimental approaches use dural application of algogenic substances to model the proposed meningeal neurogenic inflammation thought to initiate migraine-related pain via trigeminovascular afferent and central neuronal sensitization [60, 61]. Inflammatory substances can be applied to the dura singly or in combination as an inflammatory soup. Commonly used substances include histamine, serotonin, bradykinin and prostaglandin E2. Other substances including capsaicin, low or high pH buffered solutions [62], cytokines [63] and complete Freund's adjuvant [64, 65] have also been used. The application of these substances has been used to examine peripheral and central neuronal sensitization to various stimuli. Meningeal exposure to the above mentioned inflammatory substances alone or a combination as an inflammatory soup has been used as a reliable method of activating and sensitizing trigeminovascular meningeal afferents in-vivo as measured by enhanced trigeminal ganglion responses to mechanical stimulation of the meninges [61]. Meningeal application of this inflammatory soup also produces activation and sensitization of central neurons in the trigeminal nucleus with convergent dural and cutaneous receptive fields [60].

Over time, advancements in this model generated alternate delivery methods, requiring less invasive procedures that are now amenable to behavioral testing. While sensitivity of trigeminal ganglion and trigeminal nucleus caudalis neurons to mechanical stimulation following inflammatory soup infusion suggests heightened trigeminal nociception, assessment of conscious pain related reflexes in the animal offers an additional methodology for determining changes in pain perception. Response to mechanical stimulation using von Frey monofilament testing of the periorbital region in the awake behaving animal was therefore a critical evolution

of the migraine pain model (see Behavior section). To permit behavioral testing in response to chemical dural stimulation, various models have been developed to allow for administration of substances in conscious behaving animals [66–69]. Repetitive inflammatory soup administration induces a chronic periorbital hypersensitivity to tactile stimuli that lasted for up to 3 weeks, suggestive of a model of chronic migraine [70]. In selecting this model, the researcher must consider that upstream events leading to trigeminal activation are bypassed and the chemical cocktail utilized requires careful control to prevent supramaximal stimulation. The surgical procedures, while improved, are intricate and could result in mast cell degranulation around the insertion site of the catheter.

Exogenous administration of algogenic substances in-vivo

A key feature of migraine is that various triggers can initiate an attack and experimentally, chemical triggers have been used extensively in human models of migraine [71, 72]. Nitric oxide donors, including nitroglycerin, have emerged as the most prominent exogenous algogenic substances to date. This is based on early observations of their headache producing qualities in angina patients and during occupational exposure [73, 74]. However, more recently CGRP, PACAP and cilostazol have all emerged as viable human migraine triggers [75–77] and have been reverse translated into preclinical models of migraine pain [52, 58, 78, 79].

Importantly from a translational aspect, it is now becoming evident that such exogenous algogenic substances also trigger other migraine-related features in conjunction with pain responses. For example, both nitroglycerin and PACAP, but not CGRP, trigger migraine premonitory symptoms in patients [80–82] and nitroglycerin triggers cranial allodynia [52]. Recent pre-clinical studies have identified several pain-related and non-pain phenotypes following their administration and their utility is further enhanced by the ability to study both acute administration and a more chronic regime, considered relevant to migraine chronification [83].

The selection of a specific algogenic agent is dependent on the individual study requirements. Nitroglycerin and related nitric oxide donors have been used both in combination with in-vivo electrophysiological models where they induce a latent sensitization of trigeminal sensory afferents [52], and in freely behaving models where they induce increased activation of the trigeminovascular system and both periorbital and hindpaw hypersensitivity to tactile and thermal stimuli [84, 85]. As an alternate, based on the developing therapeutic potential of inhibiting CGRP signaling [8], the use of CGRP preclinically is increasing. Originally used in models of neurogenic dural vasodilation that

explored therapeutic interventions on peripheral neurovascular CGRP signaling at the level of the dura mater [86, 87], more recently CGRP has been shown to trigger photophobia, periorbital hypersensitivity and spontaneous pain behaviours in rodents [79, 88, 89]. Furthermore, PACAP has shown preclinical potential. In the hemisected skull model, PACAP-38, but not PACAP-27 induced mast cell degranulation [90], while in-vivo studies demonstrate a delayed sensitization of trigeminovascular nociceptive processing [58] following PACAP infusion in rodents.

The use of algogenic, or migraine triggering agents in preclinical models of migraine-related pain has many advantages as well as important limitations to consider. The use of specific migraine triggering agents is strengthened by the specificity of such triggers, whereby alternate related molecules including vasoactive intestinal peptide (VIP), amylin or adrenomedullin, fail to induce pain behaviours or periorbital hypersensitivity in mice [88]. Importantly, exogenous algogenic substances are not restricted to specific peripheral or central nervous system sites and as such, have the potential to act more generally in migraine-relevant structures. This has clear advantages for disease modelling with the induction of premonitory symptoms in patients [80–82], however, it raises several unanswered questions regarding potential sites and mechanisms of action that need to be determined to fully appreciate their potential. Further, specific dosing regimens need to be adopted to allow a more thorough comparison between studies. For example, wild-type mice classically respond to a dose of 10 mg/kg of nitroglycerin, whereas mice harbouring genetic mutations linked to migraine with aura have been shown to respond to much lower doses [85]. Finally, such models have the potential to explore both acute responses and a more chronic state in rodents. Repetitive dosing over time has the potential to produce a prolonged basal hyperalgesia [83], however, such responses are also observed during chronic exposure to acute anti-migraine therapeutic agents [91], highlighting the complexity of determining divergent or shared downstream signaling cascades that may represent key targets for migraine.

Experimental readouts: electrophysiology and immunohistochemistry

Electrophysiology

As detailed previously, the activation of meningeal afferents that innervate the dural blood vessels, including the middle meningeal artery and the superior sagittal and transverse sinuses, results in headache pain that is very similar to the migrainous pain [12, 92]. Therefore, activation of the trigeminovascular system has consistently been used as a model of migraine-related pain. The

trigeminovascular system includes the trigeminal ganglion, that sends primary sensory afferents to intra- and extracranial structures [93], including the dural blood vessels, and central projections to the trigeminal nucleus caudalis and the associated first and second cervical levels [94]. Second order neurons project from the trigeminal nucleus caudalis to higher order structures in the brainstem and diencephalic nuclei involved in pain processing [95–97]. As noted, several paradigms have been developed to facilitate the activation of the trigeminovascular system in-vivo and targeted electrode placement has facilitated the recording of durovascular evoked responses throughout the central nervous system. Given the importance of the meningeal afferents and their central synapses on the trigeminal nucleus caudalis, it is not surprising that the trigeminal nucleus caudalis has received considerable attention with respect to targeted neuronal activity recordings.

Electrophysiological recordings of trigeminal nucleus caudalis neuronal responses to nociceptive durovascular stimulation have been widely used as a readout of nociceptive trigeminovascular activation [45, 46, 98, 99]. The nature of the recordings from within the spinal cord dorsal horn necessitate the use of a laminectomy of the first cervical vertebrae along with an incision of the dura mater. Recording electrode placement is largely optimized via the mapping of cutaneous and dural receptive field responses, and once the appropriate level is located, specific cell types can be identified [18]. Second order trigeminothalamic projection neurons receive the majority of their inputs from thinly myelinated A δ - and unmyelinated C-fibres [60, 100–103] arising in the trigeminal ganglion. As such, both fiber latency responses can be recorded and analyzed differentially to determine specific effects [104]. In addition to the specific A and C-fiber latencies, a variety of neuronal subtypes can be identified using high impedance electrodes that allow for single-cell responses to be recorded. The three major classes include low-threshold mechanoreceptors responding to innocuous stimulation, wide dynamic range neurons responding to both noxious and non-noxious stimuli, or nociceptive specific neurons that only respond to noxious input [105]. In addition to the trigeminal nucleus caudalis, in-vivo electrophysiology has the potential to map neuronal alterations in several migraine-relevant nuclei, with the thalamus also receiving considerable attention [106, 107] highlighting potential therapeutic benefits of modulating thalamocortical signaling.

In addition to the ability to target specific nuclei throughout the brain, direct trigeminal nucleus caudalis recording is commonly combined with alternate methodologies, including microinjection in discrete brain areas to discern functional connections. By combining

these methodologies several modulatory networks regulating trigeminal nucleus caudalis durovascular evoked responses have been identified, including the A11 [108], the locus coeruleus [47], the ventrolateral periaqueductal gray [109, 110], thalamic [57] and hypothalamic [45] nuclei. Additionally, in seminal studies exploring the impact of environmental stimuli, potential mechanisms underlying light-induced exacerbation of durovascular nociceptive processing were identified in the posterior thalamus [111].

This in-vivo model has proven highly predictive in the pharmacological screening of potential antimigraine compounds. Experimental pharmacological evidence has shown that effective treatments such as triptans [112–114], CGRP antibodies [104, 115], gepants [116], lasmiditan [117] and vagal nerve stimulation [118] all demonstrated significant efficacy; compounds that have failed clinical trials such as neurokinin 1 receptor antagonists do not [59].

A particular method to characterize the pharmacology of neuronal responses is the use of in-vivo electrophysiology in combination with microiontophoresis [119]. In this setup, a multi-barrel electrode, that includes a recording electrode and several capillaries, is used to pharmacologically modulate the neurons, that at the same time are being recorded by using the flow of electric charge through an aqueous solution to eject drugs to a small number of cells. Microiontophoretic ejection of ergot alkaloids [120] and triptans [121–123] in the trigeminal nucleus caudalis has been shown to inhibit nociceptive durovascular and local glutamate-evoked responses indicating a potential central action of these compounds. Interestingly, the later glutamatergic based direct activation may allow for a degree of site specificity to be defined, as the exogenous glutamate likely acts on postsynaptic receptors and thus an ability to selectively block this response would suggest a postsynaptic effect on trigeminothalamic projection neurons and not a direct effect on incoming meningeal afferents. This approach has identified potential central sites of action for several anti-migraine therapeutic targets including the CGRP receptor antagonist olcegepant [124] and the 5-HT_{1B/1D} receptor agonist naratriptan [107]. While the ability to determine potential local action of specific compounds is an advantage, it relies on bypassing the blood brain barrier that remains a significant barrier to the clinical development of central nervous system targets.

The use of in-vivo electrophysiology has several advantages, not least of which is the flexibility of methodologies to activate the underlying pathways of interest, including specific dural evoked responses and those elicited following the administration of exogenous algogenic substances. However, great care must be taken during

surgical procedures and while modeling migraine-related pain in an intact nervous system with complex interfaces between the peripheral and central compartments is an advantage, the invasive nature of the surgery and subsequent disruption of the blood brain barrier must be carefully controlled for. Finally, current in-vivo electrophysiological procedures are largely acute in nature and therefore preclude longitudinal studies; future research should and most likely will take advantage of the increasing trend to conduct such studies in conscious behaving animals [125].

Immunohistochemistry

C-Fos immunoreactivity

A complementary or alternate method to in-vivo models of migraine-related pain is the identification of neuronal activation in key nociceptive processing structures such as the trigeminal nucleus caudalis using markers of neuronal activation, mainly c-Fos immunoreactivity [126]. The gene FOS is an immediate early gene that encodes the proto-oncogene c-Fos, which dimerizes with transcription factors of the Jun family to build up the transcription factor AP-1 regulating the expression of downstream target genes [127]. In neurons, c-Fos expression can be stimulated by at least 3 second-messengers, including cAMP, protein kinase C and calcium-calmodulin, through the activation of the CREB/Cre complex [128]. c-Fos expression can be detected from 30 min to an hour after intense stimuli, reaching its peak at 2-4 h and returns to basal levels 8-24 h following stimulation [129]. Most commonly, c-Fos expression is visualized via the immunohistochemical detection of c-Fos in the nuclei of cell bodies [130].

c-Fos was one of the first transcription factors whose induction was shown to be activity-dependent [131]. Early studies identified that c-Fos is induced in the spinal dorsal horn following peripheral noxious stimulation [132], leading to its widespread use to study nociception [133]. In migraine-related pain research, c-Fos expression is commonly used as a valuable tool to identify subpopulations of neurons activated in response to noxious stimuli and related nociceptive pathways [19]. Hence, many studies have used c-Fos immunoreactivity to map neuronal activation throughout the trigemino-vascular system, which has helped to generate a greater understanding of migraine pathophysiology [49, 113, 134–136]. Electrical, mechanical and chemical stimulation of meningeal afferents and systemic administration of algogenic substances including nitroglycerin induce c-Fos expression in the nociceptive-specific laminae of the trigeminal nucleus caudalis [49, 134, 137–139], which can be inhibited by anti-migraine treatments such as triptans [55, 84, 99, 140, 141], dihydroergotamine [99] and lasmiditan [142]. Thanks to the ability of c-Fos to

respond to polysynaptic activation, this method also allows functional pathways to be mapped and therefore to determine ascending and descending pathways involved in migraine pathophysiology. To this end, c-Fos expression has been mapped in several brainstem structures, including the PAG [143–145], parabrachial nucleus and locus coeruleus [145]. Higher order diencephalic nuclei including the hypothalamus (e.g. the ventromedial nucleus, the supraoptic nucleus and the posterior hypothalamus [48, 51]) and thalamus (e.g. the thalamic reticular and centromedian nuclei [146, 147]).

The use of c-Fos expression has facilitated stepwise changes in our understanding of the pathophysiology of migraine and migraine-related pain. However, researchers must be wary of specific limitations, including the stimulus used to drive its expression [19]. This limitation is shown by the failure of substance P-neurokinin-1 receptor antagonists in acute and preventive treatment of migraine [148], although they have been shown to block c-Fos expression in the trigeminal nucleus caudalis following trigeminal ganglion stimulation [141, 149]. Importantly, the lack of c-Fos expression does not guarantee the absence of neuronal activation, as not all activated neurons express c-Fos, including those in the dorsal root ganglia [132]. Another important consideration is that the induction of quantifiable levels of c-Fos requires a strong consistent stimulation that is not usually physiologically relevant.

Alternative markers of neuronal activation

In certain conditions and with respect to specific tissues that do not express c-Fos, alternate markers of neuronal activation can prove beneficial. The extracellular signal-regulated kinase (ERK) is a member of the mitogen-activated protein kinase family. Once activated, phosphorylated ERK (pERK) is translocated into the nucleus where it activates several transcription factors [150]. Like c-Fos, pERK expression [151] is very robust, requires high-threshold noxious stimuli and can be inhibited by analgesics. Unlike c-Fos, pERK expression is faster and more dynamic, it cannot be induced by innocuous stimuli and it is found in most subtype of neurons, including dorsal root ganglia neurons [152], as it is summarized in Table 1.

Experimental readouts: behavioral assays

Measuring pain-like behaviours in awake, freely-behaving animals can provide key insights into the complex and integrative systems underlying migraine-like pain. An advantage of assessing pain-like behavior in conscious animals is the ability to assess the impact of experimental manipulations or therapeutic interventions on the whole animal. However, in doing so, it is

Table 1 Comparison of several features of c-Fos and pERK expression (Adapted from [152]). IHC, immunohistochemistry

	c-Fos	pERK
Methods of detection	IHC	IHC
Mechanism of detection	Gene expression	Phosphorylation
Induction by noxious stimuli	Yes	Yes
Induction by innocuous stimuli	Sometimes	No
Stimulus intensity-dependent	Yes	Yes
Subcellular distribution in neurons	Nucleus	Nucleus, cytoplasm, dendrites, axons
Time course after formalin injection		
Onset	30 min-1 h	1-3 min
Peak induction	1-2 h	2-10 min
Return to base	8-24 h	1-2 h

imperative that the experimenter is blinded to treatments/experimental groups, as many behavioural assays can be subjective and therefore vulnerable to unconscious bias. When properly controlled, behavioural readouts are an invaluable tool for investigating migraine-like pain phenotypes underlying migraine pathophysiology.

Behavioural assays modeling migraine-like phenotypes can exploit sensory discriminative/evoked pain-like behaviors that focus on the trigeminal/spinal reflexes, operant models that assess cognitive aspects of pain, as well as spontaneous, non-evoked pain behavior. This section will focus on sensory discriminative readouts, as they are the most common and easily quantifiable, but will also touch briefly on other pain-like models as well.

Modeling cutaneous allodynia

Sensory discriminative models in headache research tend to exploit a common associated symptom of migraine: cutaneous allodynia. Allodynia is defined as the perception of normal innocuous sensory stimuli as uncomfortable or painful. It has been reported that 70% of migraineurs experience cephalic allodynia: referred pain or sensitization around the head that is induced by the activation of the trigeminal system during an attack [153]; extracephalic allodynia in the arms and legs is reported in more severe and chronic cases [154, 155] and is likely attributed to sensitization of third order trigeminal neurons in the thalamus [153, 156]. As such, measurements of mechanical and thermal sensory nociceptive thresholds as a readout of cutaneous allodynia can be a reliable marker for migraine pathophysiology.

Mechanical allodynia

The most commonly used behavioural assessment of pain-like behavior in preclinical headache models is mechanical allodynia. Mechanical, or tactile, sensitivity is easily quantified by using calibrated von Frey filaments. These filaments are typically applied to the cephalic (whisker pad or periorbital areas) or extracephalic (hind paw) regions to determine evoked response thresholds. There are three widely used methods for how to apply the filaments and calculate a withdrawal response: the up-down method, ascending stimulus, and percent response rate. The up-down method calculates the threshold to illicit a response in 50% of the animals based on a statistical formula [157, 158]. This method involves applying the filaments in a pattern based on the animal's response to the previous filament. If there was a positive response, the next filament applied would be the next one of less force; if there was a negative response, the next filament applied would be the one of next highest force. This would be repeated for five applications from the first positive response and a 50% mechanical withdrawal threshold calculated [157]. The ascending stimulus method sees filaments applied with increasing force until a withdrawal response is evoked and the force of this filament is recorded as the mechanical withdrawal threshold [159]. Percent response sees filaments of varying forces applied in ascending order 5–10 times and the number of positive responses to each filament are recorded and the percent response calculated [157, 160].

In preclinical headache research, allodynia is often assessed in response to dural inflammatory soup application or the administration of algogenic substances. As discussed previously, the most common and well established experimental migraine trigger is nitroglycerin. Preclinical studies commonly use nitroglycerin to sensitize the trigemino-vascular system and a single dose (1-15 mg/kg) is known to induce mechanical allodynia that can last up to 4 h in rodents [83, 84, 88]. This increased sensitivity is therapeutically responsive to triptans [83, 84] and therefore strengthens this as a model of migraine-related pain. Preclinical investigations can thus utilize acute nitroglycerin administration to assess allodynia, investigate underlying mechanisms, or assess the efficacy of novel treatment targets by determining their ability to rescue nitroglycerin induced pain-related phenotypes. Transgenic mice, harboring a human mutation in casein-kinase 1 delta, which is involved in regulating the molecular biological clock and has been linked to migraine in humans, have been shown to have altered hind paw mechanical sensitivity in response to nitroglycerin compared to controls [85]. As such, specific migraine-relevant genetic mutations appear to increase the sensitivity to nitroglycerin, which is in contrast to the previously identified inability of nitroglycerin to trigger attacks in familial hemiplegic migraine patients [161].

In addition to acute behavioural responses, repeated administration of nitroglycerin regimens have been established in order to assess biological mechanisms involved in migraine chronification. By repeated dosing, every other day for 9 days, a progressive and sustained basal hypersensitivity is observed in addition to the acute post treatment responses [83]. This basal hypersensitivity can be blocked by migraine preventives such as topiramate and propranolol [83, 162], supporting this as a model of chronic migraine and therefore be used to test novel anti-migraine preventatives. For example, ghrelin has been shown to attenuate nitroglycerin induced nociception by rescuing mechanical sensitivity, thus providing evidence that ghrelin has a modulating effect on central sensitization [163]. The basal hypersensitivity induced following chronic nitroglycerin is accompanied by increased CGRP expression in central brain areas with possible interaction with GABA and glutamate transmission that may contribute to the induction and maintenance of central sensitization [164]. Furthermore, direct stimulation of the nitric oxide receptor soluble guanylyl cyclase can chronically increase basal hypersensitivity which is subsequently blocked by acute and preventative migraine medications such as triptans and topiramate, thus indicating that nitroglycerin may in part cause migraine-related pain through stimulation of this pathway and that activation of this receptor may be an important component for the maintenance of chronic migraine [165].

In addition to nitroglycerin, other migraine provoking substances have been seen to elicit cephalic and extracephalic allodynic responses in rodents. Acute administration of CGRP, PACAP, histamine and prostaglandin E2 were shown to elicit periorbital mechanical sensitivity, which was attenuated by systemic antagonists [88]. Furthermore, intrathecal injection of CGRP has also been shown to induce hind paw mechanical allodynia in wild type mice and this response was further enhanced in transgenic mice that overexpress the CGRP receptor activity modifying protein 1 [166].

Finally, mechanical allodynia has been seen in response to trigeminal sensitization through other models such as inflammatory soup and cortical spreading depression. Application of inflammatory mediators onto the dura mater in awake, freely moving rats induces both facial and hind paw mechanical allodynia [67, 68, 70, 167], which is reversed by sumatriptan and CGRP receptor antagonist [67]. Cortical spreading depression, the electrophysiological correlate of migraine aura, can also activate trigeminal pain pathways. Cortical spreading depression induced mechanical allodynia has been observed in both the face and the hind paws of rats following multiple events [168]. Reduction in mechanical withdrawal thresholds ipsilateral to the cortical spreading depression can be reversed by a CGRP receptor antagonist [169].

Thermal allodynia

While most studies seem to assess mechanical allodynia, thermal allodynia (both hot and cold) can also be observed in preclinical models and can complement mechanical sensitivity to further dissect underlying mechanisms.

Cold sensitivity can be measured with ease in both cephalic and extracephalic body regions using the acetone evaporation test. Here, nociceptive behaviors are induced by the evaporative cooling of acetone on the skin and such behaviours can be counted, timed or scored [170, 171]. Assessing extracephalic thermal allodynia is easily assessed using the Hargreaves or tail flick test. The Hargreaves test involves directing a heat stimulus to the animals hind paw and measuring the withdrawal latency [172]. Additionally, a heat stimulus can be directed to the animals tail and withdrawal latency recorded. The heat stimulus can be in the form of an infrared beam or hot water bath ($48 \pm 5^\circ\text{C}$). Using the Hargreaves assay, acute nitroglycerin [84] and chronic administration of algogenic substances increased thermal sensitivity [83, 165]. Furthermore, a different model of chronic migraine, nasociliary nerve ligation in rats, exhibits a lateralized sensitization to acetone following nitroglycerin in the forehead ipsilateral of nerve ligation [173]. One important differentiation is that behavioral studies have also shown differential response to mechanical and thermal allodynia, highlighting the complexity of trigeminal pain processing. For example, Brennan et al. showed that a higher dose of nitroglycerin was required to elicit differences in thermal allodynic responses in transgenic mice compared to a lower dose required for mechanical sensation [85]. In addition, Kim et al. showed differential mechanical and thermal sensitivities in the orofacial region and hind paw following chronic nitroglycerin [174]. In this study, cold was assessed on the face via acetone, while heat on the paw, likely due to the difficulty to direct a thermal stimulus to the orofacial region of an awake, freely moving animal.

One way this can be overcome is by the use of a novel operant behavioral assay using the orofacial pain assessment device (OPAD). Here, animals are trained to drink a reward while forced to place their face through temperature controlled thermal pads. Pain is assessed as a reduction in amount of reward consumed (quantified by number of licks) as well as contacts against the thermal pads [175, 176]. Recent work has shown that nitroglycerin treatment can decrease the amount of licks/contacts in wild type mice [177] indicating an increased sensitivity to thermal orofacial stimulation.

Operant models for assessing cognitive aspects of pain

Operant pain assessment assays have been used in preclinical headache research to assess emotional

dysfunction and affective-motivational components of pain. The advantage of operant based tests compared to other evoked sensory discrimination tests is that it is also a measure of higher order pain processing rather than relying on spinal reflex-based nociception. The orofacial pain assessment device mentioned above is also a readout of the emotional or motivational component of pain in that the animal needs to choose between a reward associated with a painful stimulus or forgoing the reward in order to avoid the pain [175], which adds translational value as it does not solely rely on the reflexive component of pain.

Another operant assay is the conditioned place aversion test, which measures the amount of time the animal spends in an area that has been associated with an aversive or painful stimulus. Chronic nitroglycerin has been shown to induce place avoidance, where animals learn to spend less time in the chamber associated with nitroglycerin and thus the painful experience. Novel therapeutic targets can prevent the conditioned place aversion either through analgesic effects or stimulating reward pathways [163, 178].

Spontaneous pain behaviors

Spontaneous, or non-evoked behaviors can also be used as alternative readouts of pain and can be more indicative of headache-pain rather than the associated symptom of allodynia. Spontaneous behaviors such as exploration, locomotor activity, rearing or food and water consumption are thought to be general measures of a rodent's overall wellbeing and can all decrease with pain. Other behaviors, such as freezing and grooming can increase and thus such behaviors can be measured as indirect markers of a pain-like state [179]. In migraine headache, activation of the trigeminovascular system can lead to headache worsened by activity, so freezing in rodents might reflect an activation of this system and a defense mechanism to restrict movement exacerbation of pain [169, 180]. Grooming reflects increased attention to the affected area, which may infer pain or discomfort. The above behaviors can be measured by observation or through a behavioral analysis system applied to a standard cage that is able to detect and classify behavioral variables based on vibrations produced by the movement of the animals. In headache research, it has been shown that cortical spreading depression can induce spontaneous pain behaviors such as freezing and grooming in freely moving rats and mice [169, 180, 181] which can subsequently be alleviated by a CGRP antagonist [169]. Additionally, trigeminal activation through the application of inflammatory mediators has also been shown to decrease activity and increase resting and grooming behavior which were then attenuated by a triptan [182].

Future perspectives

While the above-mentioned models have significantly enhanced our understanding of migraine pathophysiology, led to the development of novel therapies and forged a path for future translational research in migraine; there remains an ongoing requirement to refine existing and generate novel models of migraine. While not covered here, such models should explore not only migraine-related pain, but attempt to explore alternate migraine-related phenotypes to better recapitulate the disorder as a whole.

Advanced genetic modelling

Improvements in genome wide analysis studies have led to a wealth of data on polygenic risk factors for migraine with approximately 40 genetic loci identified [183]. This ever increasing list of risk factors is now combined with several rarer monogenic mutations responsible for specific migraine phenotypes [85, 184–186]. With advances in CRISPR/Cas9 technologies to facilitate gene editing in mice [187], the migraine field now has the potential to determine the specific impact of knocking in or out specific genes of interest. Such approaches have already proven effective. For example, generation of transgenic mice that overexpress the human receptor activity modifying protein 1 essential for the canonical CGRP receptor has facilitated several studies exploring CGRP hypersensitivity. These transgenic mice demonstrate clear nociceptive hypersensitivity to the algogenic substance CGRP combined with a photophobic phenotype suggesting a potential role for increased CGRP in the generation of photophobia [188, 189]. More recently a novel circadian related mutation resulting in the loss of function of casein kinase 1 delta and subsequent PERIOD-mediated phase advancing of the circadian clock has been identified [85]. In humans this mutation results in a familial advanced sleep phase and an extremely high penetrance of migraine with aura. Generation of a transgenic mouse that harbors the human mutation, enabled the identification of specific migraine phenotypes including a reduced threshold for triggering cortical spreading depression and an increased sensitivity to the human migraine trigger and algogenic substance nitroglycerin compared to wild type littermate mice. Such studies have the potential to enhance our knowledge on the impact of genetic and genetic/environment interaction's on migraine susceptibility. In addition to specific gene editing approaches, an inbred rat model of spontaneous trigeminal allodynia has been described that is responsive to acute and preventive migraine therapies [190]. This model has recently been further inbred to generate a sustained trigeminal hypersensitivity that is responsive to acute migraine therapies with potential implications for modelling chronic migraine [191].

Advanced viral vector approaches

The neuroscience field has experienced a vast expansion in the capability to selectively target specific neuronal populations using viral vector approaches. Such approaches allow for the targeted delivery and transfection of neurons based on their neurotransmitter profile, genetic makeup or anatomical distribution, while alternate transgenic mouse lines have been generated that specifically express chemogenetic and optogenetic constructs. While the use of such tools is in its infancy in migraine research, several studies have emerged that show the potential of such methodologies. For example, as discussed above, many current methods to activate the trigeminovascular system involve invasive cannula or electrode placement with potential impacts on cerebrovascular physiology. In a seminal study in the field, Houben et al. used optogenetic stimulation to activate channelrhodopsin-2 ion channels resulting in the activation of layer 5 cortical neurons and the subsequent induction of cortical spreading depression [192]. While this study primarily focused on the cortical spreading depression, it is appreciated that cortical spreading depression can act as a trigger to activate the trigeminovascular system [193] that is responsive to preventative migraine therapies [115] and trigger pain-related responses in rodents. As such, the combination of novel targeted optogenetic or chemogenetic approaches to evoke migraine-related pain and associated phenotypes in rodents holds enormous potential for the field. This approach is further enhanced by the ability to map detailed neuronal projections throughout the nervous system. More general tracing technologies have already been used to great effect in preclinical models of migraine-related pain including the exploration of pain processing pathways and potential photophobic and autonomic responses to light [194–197]. By utilizing novel tracing technologies that allow unrivaled precision down to the level of the single monosynaptic inputs on a specific cell type [198] the potential to map as yet unappreciated functional migraine-relevant brain networks holds significant potential.

Conclusion

Our understanding of migraine-related pain processing and the development of novel therapeutics for its modulation has evolved via key translational research streams in humans and experimental animals. Herein, we have largely focused on the most commonly used preclinical models of migraine-related pain; however, despite significant success, there remains a major unmet need to generate novel knowledge on the underlying mechanisms of migraine initiation, associated symptoms and ultimately attack cessation. Future preclinical research is

a necessity to achieve this goal, and while alternate read-outs that can help to reduce the use of animals in research are encouraged, for now the whole animal is the only model that can accurately examine the complex interactions between the peripheral and central nervous systems. As such, it is essential that researchers continue to refine existing and develop novel models to enable studies that explore the diverse symptomatology of migraine, while continuing to achieve significant translational success in terms of novel disease modifying therapies.

Acknowledgements

Not applicable.

Authors' contributions

All authors wrote and edited the manuscript and P.R.H edited the final version for submission. All authors read and approved the final manuscript.

Funding

The study received support from the FP7 program EUROHEADPAIN (grant agreement no: 602633) of the European Union. The Wellcome Trust (grant number 104033), The Medical Research Council (grant number MR/P006264/1), the International Headache Society and the Migraine Trust. M.V-P was an International Headache Society Fellow and L.C.S is a part-funded Migraine Trust PhD student.

Availability of data and materials

Not applicable.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Received: 9 May 2019 Accepted: 19 August 2019

Published online: 29 August 2019

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