Chapter 2
Animal Models of Migraine

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The headache research field is privileged to have in its preclinical laboratories well-established animal models that significantly facilitate and improve our understanding of headache mechanisms, in particular in terms of the molecular signalling and brain networks involved. A variety of pharmacological screening approaches for novel therapeutics and for the improvement of advanced pharmacological agents can be achieved in translational research utilising these models. The available migraine models have been developed based on our understanding of migraine from clinical, migraine patient-specific evidence. These clinical phenotypes have been successfully employed to model features of the disease physiology in animals and to provide reproducible meaningful physiological measures in the laboratory.

2.1 Animal Models of Migraine

2.1.1 What Defines an Animal Model?

Any disease model, in humans or animals, needs to fulfil three essential criteria: (1) provide trustful replication of disease physiology, (2) demonstrate good efficacy of known disease treatments and (3) demonstrate a lack of efficacy of clinically known unsuccessful disease treatments. The end goals of the development and use of

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animal models are to enhance our understanding of disease mechanisms and to aid the discovery of novel anti-migraine treatments.

Migraine is the most well-studied type of the primary headache, and the animal models used in migraine research have proven their modelling potentials in terms of screening clinically effective and ineffective treatments. Their major disadvantage, however, lies in their ability to replicate a complete disease phenotype, although this does not necessarily hamper their use in drug discovery or pathophysiology studies. Migraine is a complex neurological disorder that involves specific characteristics, spontaneous or biologically (stress, lack of sleep and missing meals are few of the well-recognised migraine triggers) triggered episodic attacks, characterised by intense headache that becomes worst with movement, accompanied by nausea, photophobia, phonophobia or even osmophobia and occasionally aura [1]. Outside the headache and aura phase, the attack onset appears to occur earlier during the premonitory phase [2]. Even in the interictal phase, migraine sufferers may have a differential processing of sensory information, as indicated by their lack of habituation to normal stimuli [3]. The migraine animal models are somewhat limited as they model aspects of the migraine syndrome and not the entire spectrum of symptoms. Currently, no animal model exists that replicates all components of migraine, particularly the sensory disturbances seen during the attack, as well as the premonitory phase events and the lack of habituation. This disadvantage largely reflects the lack of understanding we have on the migraine pathophysiology itself, and it is partly compromising the human models of migraine too. However, despite their inability to model the full spectrum of migraine, the migraine animal models are considered among the most successful neurological disease models, as they do model aspects of the disease and are indeed reliable tools for pharmacological investigations. As a comparison, the widely used cerebral infraction model (middle cerebral artery occlusion model) in the field of stroke research fails to prove the efficacy of the sole clinically available treatment, the tissue plasminogen activator [4].

Animal models of migraine are mostly acute system activation models. Acute migraine models are more widely used and are divided into three large categories with regard to the aspect of migraine pathophysiology they model: (A) Models of activation of the involved pain pathway, the trigeminovascular system – the trigeminal nerve innervation of dural structures, mainly blood vessels, and the trigeminal ganglion. This model mainly reproduces the process of events that are thought to at least replicate components of the pain perception that occurs during the headache phase of a migraine attack. (B) Models of cortical spreading depression that reproduce the possible events occurring during aura. (C) Models of nitric oxide (NO) signalling (provocation models). This model is based on clinical studies establishing that NO donors can trigger a migraine attack in sufferers after a delay of hours [5] and even reproduce premonitory symptoms and nausea in some patients [6, 7]. Neither in humans nor in animals NO donors induce a migraine aura or attenuate cortical excitability. Additionally, the identification of monogenic mutations as the cause of rare types of migraine has further allowed the development of genetic models with knock-in mutations in their genome [8]. A combination of the above modelling assays in these genetically modified animals has been further employed to

answer the enigma of migraine neurobiology. In the past few years, some attempts have been made to develop chronic, conscious migraine models; however, the characterisation of the produced phenotype, as well as their efficacy to migraine treatments, is yet to be validated among different laboratories.

2.1.2 Ethical Considerations

The use of animals in experimental research is under strict regulatory control by different authorities. The considerations around the use of animals in medical research evolve around three directions, known as the 3Rs: reduction, measures to ensure that the minimum number of animals will be used; refinement, how to achieve objectives with minimum animal suffering; and replacement, how to achieve the same objectives without using animals. Any research design should justify the 3Rs. Migraine is a complex disease, and the determination of neuronal changes not only requires the presence of neurons at a state of nociceptive condition but also requires intact brain pathways that influence each other. As such, pathway investigations cannot be ethically conducted in humans; there is no feasible alternative that would entirely replace the use of living animals that would allow the objectives to be met. Good laboratory practices should be used, however, to minimise animal suffering and to reduce the number of animals used. The majority of migraine models have been thus developed in anaesthetised animals, in which suffering is considered minimum. In preclinical research in general, however, there is growing concern that poor experimental design and lack of transparent reporting contribute to the frequent failure of preclinical animal studies to translate into treatments for human disease. In 2010, the Animal Research Reporting of In Vivo Experiments (ARRIVE) guidelines were introduced [9–11], and adapted by many regulatory authorities and medical journals, to help improve reporting standards. The guidelines refer to common good laboratory practice and should be seen in the same perspective as the guidelines established for clinical trials in humans.

2.1.3 Migraine Pathophysiology

Migraine pathophysiology is extensively analysed elsewhere in this book; however, for the purposes of better understanding the neurobiology utilised in animal models, a brief description is given below.

The migraine headache is perceived to be felt on intracranial structures, such as the dura mater and intracranial vasculature [12]. The sensory innervation of these structures arises from the trigeminal nerve, mainly from unmyelinated C-, and thinly myelinated Aδ-fibres, which have their cell bodies in the trigeminal ganglion. Nociceptive activation of these trigeminal fibres is referred to as “trigeminovascular activation” [13]. The trigeminal fibres that transmit sensory information from intracranial structures synapse on second-order neurons within the trigeminocervical complex (TCC; trigeminal

nucleus caudalis, C1 and C2 spinal levels). These neurons give rise to the main ascending trigeminothalamic pathway that relays sensory information to third-order neurons in the contralateral thalamus. The thalamus, mainly the ventroposteromedial thalamic nucleus (VPM) and the posterior thalamic nucleus (Po), is acting as the last gate before sensory information is transmitted to cortical areas involved in the processing of pain perception. A complex of descending networks from multiple brainstem, midbrain and cortical nuclei modulate the excitability of the ascending trigeminothalamic pathway [14]. In the absence of any evidence of malfunction in the trigeminovascular system [13, 15], a disruption of normal endogenous descending modulatory tone in the brain may play a critical role in migraine. However, what really alters the excitability of the ascending trigeminothalamic pathway, in a manner that a migraine attack may develop in susceptible individuals, remains to be revealed.

The migraine aura is now believed to result from the neurophysiological event called cortical spreading depression (CSD) [16]. CSD is a wave of cortical neuronal depolarisation, followed by depressed activity and associated with blood flow changes [17]. In migraine patients, CSD is believed to spread out from the occipital cortex, but it remains enigmatic how CSD is triggered in patients during migraine aura.

Accumulating evidence exists as to why the trigger of migraine attacks should be sought in the hypothalamus [18]. The strongest, direct evidence for hypothalamic activation in migraine patients arises from brain imaging studies. These studies demonstrated, using positron emission tomography (PET), increased blood flow in the posterior region of the hypothalamus during the very early stages of spontaneous migraine attacks [19] and during the premonitory phase of the NO donor, nitroglycerin (NTG)-induced migraine attacks [20].

2.2 Models of the Peripheral Trigeminovascular System

Animal models investigating changes in the peripheral branch of the trigeminovascular system, i.e. the dural environment and vasculature and the trigeminal fibres innervating these, are believed to model peripheral events that are likely to occur during a migraine attack. As our understanding of migraine has progressed over the years, some of these models are now considered redundant, particularly those demonstrating a pure vascular site of action. Nevertheless, their utilisation in the triptan era, as well as the lessons learned from them, makes knowledge around these models an integrated facet of the scientific progression in the field.

2.2.1 Vascular Models

Vascular animal models had been developed during the first epoch phase of scientific exploration in migraine models and were based on the view that extracerebral vasodilation could occur during a migraine attack. Indeed, successful migraine

treatments include drugs which do not cross the blood-brain barrier (BBB) and evoke vasoconstriction, such as sumatriptan and ergot alkaloids [21]. Identification of elevated levels of the vasodilatory peptide calcitonin gene-related peptide (CGRP) in patients during a migraine attack [22] further reinforced, at the time, the theory of an integrated vascular involvement in migraine pathophysiology. However, a role for cephalic vessels in the development of migraine as a syndrome has been criticised over the years. Magnetic resonance angiography data during NTG-triggered migraine attacks suggests no association with vasodilation of cerebral or meningeal vessels [23]. It is now well established that not all vasodilatory peptides trigger a migraine attack [24], while following intravenous infusion of other vasodilatory peptides, a migraine attack is triggered hours after the cease of the vasodilatory effects [25]. Vasodilation alone may be an epiphenomenon of migraine attacks, which is not sufficient to induce pain. Additionally, not all vasoconstrictive therapies alleviate migraine symptoms, while many of the clinically effective anti-migraine drugs do not have a vasoactive action [26]. Nevertheless, the use of vascular models in migraine research made it clear that it is not the vasodilation, as such, that is important in migraine pathophysiology but the induction of second messenger signalling pathways that vasoactive substances induce through their interactions with G-protein-coupled receptors. Vascular models have thus allowed the collection of reliable information for the vasodilatory role of neuropeptides found in the trigeminal nerve endings, measurement of intracellular calcium changes and second messengers' concentration.

Nevertheless, the vascular models of migraine are worth referring to, not only due to their use during the triptan era but also for their usefulness in evaluating the vasoconstrictive profile of potential anti-migraine therapeutics, which can be contraindicated for some patients. In combination with immunocytochemical methods on isolated cerebral vessels, the anatomical localisation of receptors, such as of 5-HT_{1B} and CGRP receptors, had become more clear [27, 28]. Additionally, with the new development of CGRP antibodies [29–31], the models may be found once again useful in identifying the long-term effects of vasodilation blockade (due to CGRP) on the actual vascular bed.

2.2.1.1 Constriction of the Carotid Arteriovenous Anastomoses

This model was used in the early phases of triptans' validation as potential therapeutic agents. The model aimed to replicate clinical evidence showing that the anterior jugular oxygen saturation is reduced to the ipsilateral side of the headache during a migraine attack [32], probably due to dilation of the carotid arteriovenous anastomoses, which will reduce the available oxygenated blood through its thrust into the veins [33]. This was further thought to explain clinical observations in some patients, such as facial paleness, swelling and reduced facial temperature of the frontal vein ipsilateral to the headache. The model did not gain much interest in the field, not only due to its technical difficulties but also due to that fact that arteriovenous anastomoses do not appear dysfunctional in humans, particularly due to the

intact sympathetic nervous system. Decreased oxygenation of blood has been also supported to have a causative role in a minority of migraine patients with right-to-left shunts; however, studies support an unlikely significant role in migraine triggering or chronification [34].

The model used primarily anaesthetised pigs, in which the strong sympathetic influence is suppressed, allowing ~80 % of the total carotid blood flow to be shunted via arteriovenous anastomoses into the jugular venous circulation [35]. Alternatively, vasoconstriction of potential drugs in vasosympathectomised dogs was also employed [36]. Sumatriptan, ergot alkaloids and α-adrenoceptor agonists were shown to reduce carotid arteriovenous anastomotic shunting [21, 37]. The model has thus been used to predict a clear vascular site of action of potential treatments. Such a complicated and demanding model these days can be easily replaced by in vitro vascular preparations.

2.2.1.2 Constriction of Cephalic Blood Vessels

More direct vascular models that investigate the pure vasoconstriction properties of therapeutics were also developed both as in vivo and in vitro setups. In vitro vascular models use isolated cranial vessels (including human arteries) and isometric measures of vessel diameter in order to study the contraction or relaxation of vascular segments mounted in organ baths during application of potential anti-migraine drugs. This model has been successfully used to evaluate the vascular action of triptans and demonstrated the 5-HT_{1B} receptor efficacy of sumatriptan [38]. Using this model, Möller-Schhweinitzer and Weidmann suggested as early as in 1977 [39] that the anti-migraine efficacy of ergotamine was due to a pure vasoconstrictive action.

A variety of specific acute anti-migraine drugs, including sumatriptan and ergot alkaloids, have been shown to produce selective vasoconstriction of cephalic blood vessels in an in vivo vascular model [21], utilising initially dogs and rabbits, and rats and guinea pigs later. The vasomotor role of endogenous neuropeptides of the perivascular trigeminal nerve endings has been further studied in this model by local luminal and abluminal applications [40, 41]. Using intravital microscopy over a cranial window that allows direct measurements of the dural blood vessel diameter or blood flow, it was shown that topical or intravenous administration of CGRP induces vasodilation, which is blocked by the CGRP antagonist $CGRP_{8–37}$ [42, 43]. Exogenous CGRP acts directly on CGRP receptors on the smooth muscle of dural arteries and compounds that inhibit CGRP-induced dilation demonstrate at least their partial action on the smooth muscles of blood vessels [44]. Similarly, systemic administration of NO donors causes reproducible dural blood vessel dilation [45, 46]. A number of compounds including triptans, CGRP antagonists, cannabinoid receptor 1 (CB1) agonists and nitric oxide synthase (NOS) inhibitors have been found to attenuate this chemically induced vasodilation [45, 47].

A major limitation of the above model when studying the effects of vasodilatory peptides, such as CGRP, is the resulting hypotension and potential activation of autoregulatory mechanisms [41]. The subsequent vasodilation of the cranial vasculature

makes the involvement of an actual vasomotor pharmacological interaction or of an autoregulation mechanism activated in response to hypotension difficult to interpret [48]. An alternative to the potent hypotension induced by intravenous administration of vasodilators was suggested by Gupta and colleagues, who demonstrated that intracarotid administration of CGRP induces maximum middle meningeal artery dilation with minimum blood pressure effects [49]. This route of administration can be successfully adopted for other vasodilatory substances, including NO donors.

2.2.2 Neurovascular Models for Peripheral Investigations of the Trigeminovascular System

Neurovascular animal models aim to reflect more appropriately the involvement of the peripheral nerve fibres in the modulation of the dural vascular tone.

2.2.2.1 Neurogenic Dural Vasodilation and Blood Flow Model

The involvement of CGRP in migraine pathophysiology has been crucial in the development of experimental animal models of migraine. Migraine patients appear to have elevated levels of CGRP in the cerebral circulation during a migraine attack [22], although these findings have been criticised in other studies [50]. Using animals, it was later shown that stimulation of trigeminal nerve fibres innervating the dura mater induces the release of CGRP [51], which results in dural blood vessel dilation via CGRP receptors located on the vascular smooth muscle [46]. Further to the vasodilation, an output of trigeminovascular activation is a neurogenic, CGRP-driven, reproducible increase in meningeal blood flow [52, 53]. How CGRP release may be triggered in migraine patients is not clear, although an antidromic activation of the trigeminal system as an epiphenomenon of central mechanisms has been suggested, but not fully supported [54]. Nevertheless, the model directly activates the nociceptive pathway thought to be involved during the migraine headache phase. Thus, the vasodilatory reaction and blood flow increase of dural vessels following stimulation of the trigeminal fibres is used as an indirect indication of trigeminal system activation, and it models peripheral aspects of the migraine attack. It is important to note the correct interpretation of the model that it is not the CGRP-induced vasodilation or blood flow change as such that should be aimed to be blocked but the activation of the peripheral trigeminal fibres. CGRP itself is not known to sensitise trigeminal fibres either and thus does not contribute to nociceptive activation [55]. In this model, as vasodilation is a result of neuronal fibre activation, the model is known as neurogenic dural vasodilation (NDV) and permits the study of the peripheral branch of the trigeminovascular system.

In the NDV model, trigeminal fibre stimulation is mostly achieved through application of electrical stimulation from bipolar electrodes positioned near dural arteries, such as the middle meningeal artery, on a closed cranial window. The model utilises mostly intravital microscopy which permits the direct study of cranial blood

vessels' diameter or laser Doppler flowmetry for detection of dural blood flow changes. Electrical stimulation of the closed cranial window causes a neurogenic reproducible dilation and increase of blood flow of the underlying dural vessels, via activation of the trigeminal nerve fibres. Vessel dilation is due to CGRP release from pre-junctional trigeminal nerve endings innervating the dural vessels [46, 52, 53, 56], which binds to CGRP receptors on the smooth muscle of dural vessels resulting in vasodilation. The CGRP antagonist $CGRP_{8–37}$ is able to completely inhibit NDV, further indicating the importance of this peptide in NDV and the usefulness of modelling, at least partly, the pharmacology of the trigeminovascular system. The model is performed in anaesthetised rodents, and good laboratory practice that can assess the depth of anaesthesia, changes in blood pressure and temperature must be employed during its use, to allow for reliable outcomes to be delivered.

Beyond perivascular electrical stimulation, it was further shown that employment of chemical stimulation of the trigeminal fibres, through capsaicin, for example, could be also used to study neurogenic vasodilation. Capsaicin-induced vasodilation is elicited by the release of CGRP, as it can be blocked by a CGRP antagonist [57]. Capsaicin binds on the transient receptor potential vanilloid type-1 (TRPV1) found mostly on small diameter sensory fibres and depolarises them [58]. The induced vasodilation occurs due to the release of, among other peptides, CGRP. However, TRPV1 antagonism does not block NDV induced by perivascular dural electrical stimulation [59], indicating that electrical stimulation of the trigeminal fibres does not activate TRPV1 channels. This may further suggest that TRPV1 receptors do not play a significant role in, at least the antidromic, activation of the peripheral side of the trigeminovascular system. Additionally, although NO can itself act as a smooth muscle relaxant [60], there is evidence suggesting that NO activates trigeminal neurons by inducing CGRP release [61]. A synergistic relationship between CGRP and NO may exist, as CGRP receptor activation can increase the expression of inducible nitric oxide synthase (NOS) and stimulate NO release from glial cells in the trigeminal ganglion [62, 63]. NOS inhibitors were also effective in modulating dural blood flow [63].

This model mainly tests the action of systemic administration of potential anti-migraine compounds. Since CGRP receptor antagonists are clearly effective in acute migraine treatment [64, 65], the pharmacology of the mechanisms responsible for CGRP release from trigeminal fibres is of direct relevance to the development of newer migraine therapies, particularly for the newly described CGRP antibodies that currently undergo clinical trials [29, 30]. The model has the proven ability to predict the anti-migraine therapeutic potential of compounds, as triptans and dihydroergotamine were effective in inhibiting NDV, potentially by inhibiting the presynaptic release of CGRP from trigeminal fibres [47, 52, 53, 66–68]. The lack of an inhibitory NDV effect following neurokinin-1 receptor (NK1) antagonism, which will block the vasodilatory effects induced by substance P (SP) [66, 69], similarly to the poor results obtained with the use of NK1 antagonists in clinical trials [70, 71], further validates the good efficacy of this model. Thus, a series of compounds which include calcium channel blockers [72], cannabinoids [73], adenosine A1 receptor agonists [56], orexin 1 receptor agonists [74] and $5\text{-}HT_{1F}$ and $5\text{-}HT_{7}$

agonists [75] and nonsteroidal anti-inflammatory agents (NSAIDS) [76] that are able to inhibit NDV may indeed represent potential new migraine therapeutics.

The NDV model can be used in combination with the vascular model of CGRP infusion to compare a vascular over a neuronal side of action of potential therapeutics. Thus, the use of intravital microscopy can further facilitate dissecting the pharmacology of the trigeminovascular system and the potential site of action (vascular and/or neurogenic) of therapeutic compounds. Compounds that attenuate NDV, but not CGRP-induced dilation, are more likely to have a direct action on CGRP release from the pre-junctional site of the trigeminal fibres. Examples of such compounds include clinically active therapeutics, such as topiramate, rizatriptan and sumatriptan [44, 67], as well as potential anti-migraine treatments such as orexin 1 receptor agonists and calcium channel blockers [74, 77]. Additionally, from a biological perspective, female hormones were shown to enhance NDV through increased CGRP release from perivascular nerves and not through vascular changes, suggesting a trigeminal neuronal mechanism through which female hormones may exacerbate migraine in women [78].

Despite being proven as a highly predictive model of anti-migraine efficacy of treatments acting peripherally, the NDV model has important limitations. Clinically active compounds that have central neural system site of actions cannot be screened using this model. For example, the efficacy of many potential anti-migraine compounds that act centrally such as 5-HT_{1F} receptor agonists, dopamine receptor agonists and kainate receptor antagonists are not seen using this model [56, 68, 75, 79, 80]. Thus, caution must be used when testing compounds using the NDV model, which should be used along other models for a better understanding of the actual site of action of different therapeutics. Similarly, clinically active preventatives, such as propranolol, valproate and flunarizine, were unsuccessful at inhibiting NDV, which suggests a lack of action at the peripheral end of the trigeminal nerve [45, 56, 68]. On the other hand, as these preventatives have a clinically effective action in reducing the frequency of attacks over prolonged administration, the acute nature of the model cannot be used to investigate the long-term effects of treatments. As migraine is believed to be a disorder of the brain, the translational effectiveness of the model can be somewhat questioned.

2.2.2.2 Neurogenic Inflammation and Plasma Protein Extravasation Model

An earlier theory in migraine suggested that activation of trigeminal sensory fibres leads to sterile neurogenic inflammation characterised by plasma protein extravasation, vasodilation and mast cell degranulation within the meningeal environment. This is thought to be mediated by neuropeptide release from trigeminal sensory fibres and that it could induce pain [68, 81–83]. This theory has derived from indirect evidence mainly from preclinical studies, in which trigeminal ganglion stimulation or chemical activation of meningeal trigeminal fibres induces vascular and mast cell changes, with a concurrent vasodilation due to increased release of CGRP

and SP. Release of tachykinins and endothelin-3 further promotes vascular permeability leading to protein leakage from post-capillary venules, also known as plasma protein extravasation (PPE), and activation of dural mast cells [84]. Activation of dural mast cells will result in the release of inflammatory mediators that, along with other inflammatory mediators of neurogenic origin, such as SP and CGRP, could produce long-lasting activation and sensitisation of trigeminal nociceptors [85]. These events are in line with the occurrence of nociceptive neurogenic inflammation of the dura mater in rodents [86]. The rodent model considers inflammation to play a key role in migraine pathophysiology; however, although it is generally accepted that the initiation of a sterile inflammatory response could induce nociceptive-like behaviour in animals [87], it is not clear whether this is sufficient to induce migraine. More importantly, how neurogenic inflammation may be induced during migraine attacks cannot be reliably answered. In patients, despite the efficacy of nonsteroidal anti-inflammatory drugs (NSAIDs) in providing, at least some, pain relief of migraine headache, no PPE has been detected with retinal angiography in patients during acute attacks of migraine or cluster headache [15], further questioning the validity of the model. In this model, SP seems to be the primary mediator responsible for PPE, as gene knockout studies confirm that neurogenic inflammation is via tachykinin receptor activation on endothelial cells [88], whereas CGRP alone does not induce PPE [86]. Interestingly, in contrast to CGRP, SP levels are only moderately different during migraine attacks [89].

In the neurogenic inflammatory model, plasma protein extravasation into the meninges is mainly induced by electrical stimulation of the trigeminal ganglion. PPE is detected by measuring the amount of extravasated albumin in the dura mater, using radiolabelled bovine serum albumin or of Evans blue which can bind directly to albumin. After unilateral stimulation of the trigeminal ganglion, the dura mater is removed and the ratio of the average labelled intensity of the stimulated side compared to the non-stimulated side is calculated. Intravenous administration of serotonin and of various neuropeptides, including SP, neurokinin A and bradykinin can additionally lead to meningeal PPE. Parasympathetic activation seems to also result in neurogenically mediated meningeal PPE [90].

The hypothesis of a sterile neurogenic inflammation in migraine was supported initially by the fact that PPE can be blocked by a number of clinically active treatments, such as ergot alkaloids [81]; cyclooxygenases1/2 (COX1/2) inhibitors, including indomethacin [91, 92]; and 5-$HT1_{B/D/F}$ agonists [93–95] including sumatriptan [96]. However, the subsequent failure of neurogenic inflammation blockade to demonstrate an efficacy in a clinical setting further contributed to the unreliability of the model and the characterisation of the theory of neurogenic inflammation in migraine pathophysiology as inadequate. Neurokinin-1 antagonists which block PPE in rodents [97, 98] were ineffective in patients [71, 99], while the specific PPE blockers, CP-122,288 and 4991w93, and a number of endothelin antagonists which prevent tachykinins and endothelin-3 from inducing PPE were not effective in aborting migraine attacks in the clinic [100], despite blocking neurogenic inflammation in this animal model. For these reasons, the neurogenic inflammation model following electrical stimulation of the trigeminal ganglion and assessment of PPE stimulation is now considered redundant.

2.3 Trigeminovascular Activation Model for Central Neural System Mechanisms

Migraine is now established as a brain disorder, given the lack of clinical evidence of peripheral changes that could trigger a migraine attack [101, 102]. A number of brain areas are consistently seen to be activated during all phases of a migraine attack [7, 20, 103], suggesting an important role of CNS structures in the development of migraine pathophysiology. The field is thus moving towards investigations that will allow the development of novel CNS-based therapeutic approaches. Studying the central components of the trigeminovascular pathway and modelling of central events that are likely to occur during a migraine attack offers good potentials for the development of new, efficient therapeutics.

The trigeminovascular activation model is thought to mainly reproduce the process of events that at least replicate components of the pain perception that occurs during the headache phase of the migraine attack. It is based on the fact that stimulation of the meninges, particularly around blood vessels, induces headache-like pain in humans [104–106]. The pain arises from the direct stimulation of trigeminal fibres that innervate the meninges [106] and is a result of the activation of the ascending trigeminothalamic pathway [13]. It is reasonable to assume that such stimulation is also nociceptive in other species and will activate the same sensory pathways involved in the perception of pain during migraine. In animals, activation of the trigeminovascular system can be induced by a variety of stimuli: trigeminal ganglion electrical stimulation through bipolar stimulating electrodes positioned stereotaxically in the ganglia, chemical stimulation of the meninges by direct application of an inflammatory soup or capsaicin on the dura mater, electrical stimulation of perivascular dural areas through a bipolar stimulating electrode or even mechanical stimulation evoked by repeated von Frey filaments testing on dural structures. Although the latter two approaches have been widely used, the choice of stimulation needs to be carefully selected. The lack of an inflammatory response in humans during migraine attacks and considerations for the integrity of the BBB function following chemical stimulation make mechanical and electrical stimulation of dural sites the only model for which objective, clinical evidence exists and confirms its painful nature, although it is also a non-physiological stimulus. As discussed earlier, although it is highly doubted that a significant sterile inflammatory response occurs on the meninges during a migraine attack, some kind of local release of peripheral inflammatory markers which will induce abnormal neuronal hyperexcitability in the TCC may explain sensitisation in migraine [87].

Central activation of the trigeminovascular system in this model can be assessed with multiple assays, analysed below in detail.

2.3.1 Fos Immunoreactivity

Fos is the protein product of the immediate early gene *c-fos,* which is rapidly and transiently activated in response to several forms of stimuli, including extracellular stimuli and intracellular second messenger systems. Extracellular stimuli activate

membrane receptors and initiate a second messenger cascade that results in the upregulation of the immediate early genes, which produces transcription factors including the Fos protein. *c-fos* mRNA, which can be also detected in tissue, reaches its peak about 30–40 min poststimulus, while the levels of the nuclear protein Fos peak about 2 h poststimulation. Expression of the gene can be measured via Northern blot analysis or in situ hybridisation. The protein is normally visualised using immunocytochemical techniques. Quantification of Fos, although not accurate, is usually done through microscopic blinded counting of Fos-positive profiles and less frequently through Western blot analysis, both considered as semi-quantitative approaches.

Identification of Fos expression following trigeminovascular activation has been widely used in this model. Although Fos is not a specific marker of nociception, and other extracellular signal-regulated kinases have been implicated in mediating nociceptive signalling in the brain [107], this protein has been the choice of assessment cellular activation in a number of studies. In the field of migraine research, Fos immunoreactivity has been used to identify populations of cells activated in response to either electrical [108] or chemical [109] noxious stimulation of structures innervated by the trigeminal nerve. Fos has been shown to be expressed in the TCC [108, 110, 111] and other brain areas that could be activated with such nociceptive stimulations. For example, it was found that electrical stimulation of the superior sagittal sinus (SSS) resulted in increased expression of Fos in the periaqueductal grey [112] and in various hypothalamic nuclei, including the ventromedial nucleus, the supraoptic nucleus and the posterior hypothalamus [113]. Like other markers of cellular activation, Fos can be expressed in multiple locations as a result of polysynaptic activation. It can be thus used to map network pathways that become activated during trigeminovascular activation, and this has been one of the great advantages of using the Fos model. However, caution must be used when detecting this protein, since it is not expressed in all brain areas. For example, the basal thalamus where the ascending trigeminothalamic pathway projects does not appear to express Fos [114].

Beyond the anatomical studies, expression of Fos in the TCC, as a model of trigeminovascular activation, has been widely used for the investigation of various compounds. Triptans and ergotamine have been shown to reduce Fos expression in the TCC in response to electrical stimulation of the SSS or chemical stimulation of the dura mater [110, 115]. Interestingly, the less lipophilic sumatriptan did not have a significant effect in this model [116], thereby demonstrating a central site of action of second-generation triptans. Fos activation in the TCC seems to be dependent on serotonergic mechanisms, as serotonin-depleted rats expressed a greater quantity of Fos in response to application of inflammatory soup on the dura mater, as compared with controls [117]. Melatonin [118] and valproate [119] were also shown to reduce Fos expression in the TCC. Additionally, the CGRP antagonist olcegepant significantly inhibited the capsaicin-induced expression of Fos throughout the TCC [120]. More interestingly, neurokinin-1 receptor antagonists and PPE inhibitors had no effect over Fos immunoreactivity [121, 122], further demonstrating the potential of this model in screening possible therapeutics with a clinical benefit.

The main limitation of the Fos model is that it does not offer any further information on the type of activation, of the cell type or of the neurotransmitters involved. For example, glia cells will equally express Fos [123], as well as glutamatergic (excitatory) and GABAergic (inhibitory) neurons. Additionally, potent antinociceptive agents, such as the NMDA receptor antagonist MK-801, may also induce Fos expression in some brain regions of interest [124]. It is thus difficult to discriminate neurons activated in response to ascending or descending transmission of nociceptive or antinociceptive signals [125]. Additionally, induction of the Fos protein to quantifiable levels requires a strong consistent stimulation that may not be physiological [126] and that lack of Fos expression does not equate to lack of neuronal activity [111]. Nevertheless, its ability to respond to polysynaptic activation, enabling mapping of pathways of neuronal activation at many synapses down the line, is certainly a great advantage. Using combined immunohistochemical techniques, it can be further used for double labelling or for co-localisation studies. As the Fos staining occurs in the nucleus of the cell, it can be readily co-localised with many antigens that are expressed in the cytoplasm or on the cell surface.

2.3.2 Metabolic and Blood Flow Measurements in Central Nuclei

Trigeminal nerve activation in animals, through stimulation either of the SSS or of the trigeminal ganglion, has been shown to cause an increase in regional cerebral blood flow and metabolic activity in the TCC, brainstem regions, thalamus and frontal and parietal cortex [127, 128]. This has been considered to model changes in regional cerebral blood flow during migraine attacks, where in the headache phase cerebral blood flow may be abnormally high, often outlasting the headache phase [129]. Facial blood flow was also shown to increase during trigeminal ganglion stimulation [130], perhaps reflecting the facial and neck tenderness seen in some patients during the headache phase of migraine. Metabolic activity is measured using 2-deoxyglucose autoradiography and quantitative densitometry, while a laser Doppler probe is placed in the nucleus of interest for measuring blood flow changes. Autoradiography is not as widely used these days, given its risk. Microdialysis through an appropriate pore cannula in the region of the TCC has been also used to detect changes in neurotransmitter release [131]. In this model, sumatriptan, dihydroergotamine and *N*-Methyl-D-Aspartate (NMDA) receptor antagonism [132] have been shown to inhibit blood flow changes in response to trigeminal ganglion.

This model is now considered somewhat redundant, as in humans reductions of cerebral blood flow during migraine with acute anti-migraine treatments are not reported [133], suggesting that blood flow changes do not adequately reflect the clinical manifestation of head pain. This model served as a precursor to the electrophysiological measures of trigeminovascular activation in the central pathway [83, 128].

2.3.3 *Electrophysiological Recordings in the Ascending Trigeminothalamic Pathway*

Electrical, mechanical or chemical stimulation of dural vessels in animals, mainly of the SSS and the MMA, excites second-order neurons of the ascending trigeminothalamic pathway. State-of-the-art electrophysiological techniques can thus be used to record this neuronal activity [134]. More specifically, trigeminovascular stimulation has been shown to excite neurons in the TCC [135–138] by releasing glutamate along with CGRP from primary Aδ- and C-trigeminal fibres [139], in the thalamus, mainly the ventroposteromedial and posterior nuclei [140, 141], and more recently in different cortical regions, primarily the somatosensory S1 cortex [142]. Activated neurons in the TCC and thalamic nuclei are either nociceptive-specific or wide-dynamic range. Electrical stimulation is usually the preferred method of trigeminovascular stimulation, as poststimulus analysis of the activation latency can better determine the activation of neurons in response to stimulation of Aδ- and/or C-trigeminal fibres, while extracellular single neuron electrophysiological recordings have been used by most laboratories using this model.

The trigeminovascular model in combination with electrophysiological recordings has been proved a highly reliable model for testing a wide range of compounds, in particular of potential anti-migraine therapeutics that cross the BBB. The efficacy of compounds in this model is assessed in their ability to attenuate evoked trigeminovascular activation. Experimental pharmacological studies have shown that abortive anti-migraine drugs, such as dihydroergotamine [134], second-generation triptans [141, 143] and other 5-$HT1_{B/D}$ receptor agonists [144], act on second- and third-order neurons to inhibit neuronal activation. Systemic administrations of CGRP antagonists, which are effective in clinical trials of migraine treatment [64], were also shown to decrease the activity of neurons with meningeal input [145]. Further to predicting anti-migraine efficacy of acute treatments, topiramate, a preventive anti-migraine compound, was also effective in inhibiting trigeminovascular activity in the TCC and VPM [146].

A number of potential anti-migraine compounds and the pharmacology of their receptors have also been studied in this model. As glutamatergic transmission plays a key role in trigeminovascular nociception [147], ionotropic glutamate receptors (iGluR) [146–153] were shown to be involved in trigeminovascular nociceptive transmission and, among others, demonstrated a central mechanism of action of kainate receptor antagonists [80]. Inhibitors of the orexin hypothalamic peptides were recently shown to suppress trigeminovascular activation recorded in the TCC [154]. Pharmacological modulation by adenosine A, cannabinoid 1, TRPV1 and dopamine 2 receptors was also found to induce neuronal inhibition, without concomitant vasoconstriction, suggesting a novel avenue for the treatment of migraine [73, 155, 156].

Beyond systemic administration of compounds, the combined use of electrophysiological recordings with microiontophoresis allowed an even greater understanding of the involved pharmacology along the trigeminothalamic pathway. Microiontophoresis allows the direct application of charged compounds onto

neurons while recording their electrophysiological properties. As recordings in the TCC or thalamus following systemic administration of compounds cannot provide proof of concept on the actual site of action of test compounds, which could be local or on multiple nuclei that could modulate the ascending pathway, the employment of microiontophoresis can confine the action of compounds to second- and/or third-order neurons. Furthermore, microiontophoresis allows the dissection of the inhibition of investigational compounds upon post- or presynaptic receptors. Microiontophoretic application of ergot alkaloids [157] and triptans in the TCC [158] and VPM [141] reversibly inhibited second-order trigeminal neurons demonstrating a central action of these compounds. These studies gave impetus to the development of more brain-penetrant 5-$HT1_{B/D}$ receptor agonists [159]. Furthermore, microiontophoresis of the CGRP receptor antagonist olcegepant in the TCC reversibly inhibited SSS electrical stimulation-induced trigeminocervical activation [160], and the same was shown for the thalamus, given the presence of CGRP receptors within the VPM nucleus [161]. In addition, the clinically active preventives propranolol [140] and valproate [162], but not gabapentin, were able to inhibit responses to L-glutamate and to trigeminovascular stimulation in the VPM, while topiramate was shown to act both in the TCC and VPM by blocking kainate receptors [153].

In addition to the use of this model in pharmacological screening of potential anti-migraine compounds, a small number of studies attempted to model some aspects of the physiological properties of second- or third-order neurons with regard to convergent inputs from the periphery or from brainstem and midbrain nuclei. This can be achieved by simultaneous electrophysiological recordings of neurons of the ascending trigeminothalamic pathway, while modulating electrically or pharmacologically the activity of distal nuclei or peripheral nerves. This approach led to the demonstration of convergent inputs from trigeminal sensory afferents that innervate both dural and facial structures [137], in particular those innervated by the ophthalmic division of the trigeminal nerve over second-order neurons. Additionally, these neurons receive afferents arising from the greater occipital nerve (GON) of the C2 dorsal root, which innervate cervical structures [163]. This property of second-order neurons is considered of great importance for the efficacy of occipital nerve blocks and stimulation in the treatment of chronic migraine [164]. The convergence of trigeminal and occipital fibres on second-order neurons might be also involved in the referral of pain from trigeminal to cervical structures and contribute to the clinical phenomena of cervical hypersensitivity in migraine [138].

Direct electrophysiological recordings from trigeminocervical neurons and modulation of higher modulatory nuclei have provided important insights into migraine pathophysiology. This approach has been employed as a great limitation of the available brain imaging techniques used in humans is the lack of good spatial resolution that will allow the identification of the exact nucleus or the nature of metabolic activation involved. In PET studies, brainstem nuclei have been shown to be activated during migraine headache [165] and following successful treatment [165]. A number of studies in the trigeminovascular model have suggested that the activity of the TCC is modulated through inputs from a variety of modulatory nuclei in the brainstem, such as the PAG [166], locus coeruleus [167] and dorsal raphe nucleus

[168]. Hypothalamic nuclei have been also shown to modulate the activity of the TCC, such as the A11 dopaminergic nucleus [169] and the paraventricular hypothalamic nucleus [170]. Although the VPM and Po thalamic nuclei are also known to receive a variety of inputs form midbrain and brainstem nuclei [171], their modulation by such pathways has not been investigated in the trigeminovascular model. Importantly though, a recent study demonstrated a convergence of third-order trigeminothalamic neurons in the posterior thalamic nucleus, with axons originating in retinal ganglion cells. This convergence has been suggested to be responsible for the modulation of thalamic neurons, activated in response to trigeminovascular activation, by light [172]. The interpretation of this outcome is that the convergence of photic signals onto dural nociceptive trigeminothalamic neurons that project to the somatosensory cortex exacerbates nociceptive processing, similar to the exacerbation of migraine headache by light [172].

Allodynia is an important phenomenon seen in migraineurs [173] and it is thought to be modelled in animals using chemical stimulation of the trigeminal fibres and electrophysiological recordings. Topical application of inflammatory agents on the rat dura has been shown to induce long-lasting activation of the trigeminovascular pathway and sensitisation of trigeminocervical neurons [109]. In rats, late sumatriptan intervention [174], but not dihydroergotamine [175], was not able to reverse trigeminocervical sensitisation, suggesting that, similarly to clinical observations, triptans might not be effective after the onset of central sensitisation. Intravenous or local meningeal application of COX1/2 inhibitors including indomethacin, naproxen and ketorolac was able to block sensitisation [176, 177]. In patients, it is believed that untreated migraine attacks could result in a spread of allodynia to the other side of the head or the forearm [173], indicating the potential spread of neuronal sensitisation from second-order neurons to third-order neurons in the thalamus [173]. Sensitisation of third-order neurons was shown by Burstein and colleagues using this model [178].

Currently, the trigeminovascular activation model assessed by electrophysiological recordings is considered as the most successful model of migraine headache. Perhaps, a disadvantage of the model is the use of anaesthetised animals, in which, depending on the choice of anaesthetic, nociceptive activation of the trigeminothalamic pathway may be, to some extent, suppressed. It also assumes that a decrease of the excitatory transmission of the trigeminothalamic pathway is antinociceptive; however, in a conscious model, such differences may not be observed.

2.4 Nitric Oxide Donors' Infusion Model

NO donors, such as NTG, have been shown to trigger an early-onset headache and migraine attack in sufferers after a delay of hours [5, 179]. This biphasic headache is not reproduced in healthy subjects; however, a mild early-onset headache is often reported [180], which is also associated with decreased thresholds to mechanical nociceptive stimuli [181]. NTG has been also reported to reproduce premonitory symptoms and nausea in some patients [6]. NO donors have never however been

reported to induce a migraine aura, even in migraine with aura patients. Despite being characterised as the molecule of the year in 1992 [182] and having awarded the Nobel Prize to three American scientists for identifying its signalling pathway in the cardiovascular system, how NO triggers a migraine attack in migraineurs is yet unknown. In neurons, similar to the smooth muscle cells, NO was found to act through the stimulation of the enzyme-soluble guanylate cyclase, followed by the production of the second messenger cyclic guanosine monophosphate which activates protein kinase G. This results in the reuptake of Ca^{2+} and the opening of calcium-activated potassium channels. In the smooth muscle cells, this leads to vasodilation. In neurons, it is thought that protein kinase G may activate other transcription factors which can lead to changes in gene expression that alter the response of the cell to a variety of other stimuli.

A wide range of methods are employed in the NO donors' infusion animal model. In rodents, a NO donor is normally infused systemically at doses higher than those required to model headache in humans. The NO donor's dose, modality of administration and choice of the time of observations must be carefully controlled when adopting this model for the study of the trigeminovascular system. Over the years, this model has been developed to reflect a more representative disease phenotype and a number of different outcomes can be assessed, from immunohistochemistry to behavioural changes. Although in initial studies intraperitoneal injections were performed using enormous doses of NTG that may elicit blood pressure decrease [183, 184], a more realistic approach is now adopted where NO donors are intravenously or intracarotidly infused, using smaller doses that elicit minimum blood pressure effects [76, 184, 185].

In rodents, systemic NO donors, most commonly NTG and sodium nitroprusside (SNP), have been shown to induce Fos expression in different CNS areas, including the TCC, brainstem and hypothalamus [183, 186]. A sexual dimorphism in NTG-induced Fos expression in the TCC and in hypothalamic nuclei has been observed, with female rodents expressing a higher number of Fos-positive cells, a phenomenon modulated by estrogens [187]. Small changes in the expression of receptor and enzyme components, such as CGRP receptor subunits, the soluble guanylyl cyclase and the nitric oxide synthase, along the trigeminal system have been also reported following NO donors' infusion [188, 189]. However, similarly to the human model [190], the levels of CGRP itself do not appear to change following NTG infusion in rats [191]. Expression of the cellular activation marker Fos after any stimulus peaks at 2–4 h [107], and that is also the case in the NTG model. It is, thus, scientifically inappropriate to claim that the delayed expression of Fos in the trigeminocervical complex reflects the delayed onset of a migraine attack in humans. Nevertheless, a delayed occurrence of behavioural changes, which included the development of allodynia and hyperalgesia in freely moving animals, has been also observed following NO donors' administration [192, 193]. In a preliminary report, Akerman and colleagues have further demonstrated that NTG infusion induces a delayed increase of spontaneous and evoked firing of second-order neurons in the TCC [194]. It is thus likely that, either through local signalling pathways in the TCC or through interactions with modulatory pathways, NO alters the threshold of activation of the pain pathway involved in

headaches. In which way NO donors may also induce premonitory symptoms [6] or nausea [7], it is not yet known. Andreou et al. suggested that NO interacts with dopaminergic hypothalamic pathways that modulate the activity of the TCC [195]. Indeed, in a brain imaging study in patients who developed premonitory symptom following NTG infusion, hypothalamic activation was a prominent outcome [20]. Interestingly, it has been shown that central dopaminergic neurotransmission is required for the NO-induced activation of c-*fos* in subcortical areas [196].

Sumatriptan has been shown to alleviate behavioural changes induced by NO donors' infusion [192], as well as to reduce Fos expression in the TCC [185]. NOS inhibitors and neurokinin-1 and CGRP receptor antagonists were demonstrated to reduce the number of Fos-expressing cells in the TCC [189]. A CGRP antagonist was also found to be effective in counteracting NTG-induced hyperalgesia [193]. A role for NSAIDs has been suggested for the NO-induced cellular changes [76].

Although the NO donors' infusion model is now widely used, mostly due to the relatively uncomplicated methods that can be used to assess its outcomes, several considerations need to be addressed. In healthy volunteers, NO donors induce only a short-lasting mild headache that does not respond to triptans or aspirin [180]. As the animals we use in the laboratory are otherwise healthy, it is rather difficult to interpret that NO infusion will result in a pure migraine model, and thus, the effectiveness of sumatriptan in this rodent model is further questioned. It is likely that the increased doses administered to animals compared to humans may contribute towards the expression of a more prominent phenotype; however, this is not established. In addition, the use of Fos as a system's activation tool needs careful consideration. As discussed earlier, its expression occurs following a variety of different stimuli, not just nociceptive-specific stimuli [107]. Thus, the expression of Fos in different CNS areas following infusion of NO donors just points to the areas that are susceptible to NO signalling. Nevertheless, from a practical point of view, the model offers the opportunity to study repeated behavioural changes in non-anaesthetised animals, which appear to have similar phenotype to that developed in humans [181]. As a pharmaceutical tool, the model needs to be further evaluated with treatments that have both a positive and negative benefit in the clinic.

2.5 Animal Models of the Aura Symptoms: Cortical Spreading Depression

Migraine aura involves transient focal neurological deficits, such as visual impairment and sensory or motor function impairment, and occurs in about 30 % of migraine patients just before or during the onset of the migraine headache [197]. Occasionally, these symptoms can even occur alone without the accompanying headache. The symptoms described as the migraine aura, with a cortical spreading rate of 2–6 mm^{-1}, are believed to be the result of cortical spreading depression (CSD), first identified by Leao in 1944 [198]. CSD itself is characterised as a slow wave of neuronal depolarisation and glial activation in the cortex, followed by a short-lasting depression and

accompanied by blood flow changes. Olesen et al. [199] demonstrated in humans that aura is accompanied by a short phase of hyperaemia, followed by a slowly spreading oligaemia. It was thought that vascular changes are purely a response to metabolic changes due to neuronal discharge, but this view has been tackled by results from Brennan et al. [200], showing the possibility of a dissociation of the spread of regional cerebral blood flow (rCBF) changes and CSD. The occurrence of CSD causes profound temporary intra- and extracellular changes including pH changes, release of neurotransmitters and ionic shifts accompanied by cellular swelling [201]. CSD in the neocortex of a variety of species, including human, has been demonstrated to be dependent on activation of the NMDA receptor, and both glutamate and NMDA receptor agonists are capable of inducing CSD if applied cortically [202]. Interestingly, the NMDA receptor antagonist ketamine was tested clinically and found effective in treating the aura symptoms, but not the headache, of migraine patients with aura [203].

What triggers a CSD in patients has yet not been determined. It is thought that altered cortical excitability may be responsible for lowering the threshold of cortical activation in some patients. Such a hyperexcitable cortex may thus give rise to a CSD, but this theory needs to be further elaborated. In animals, CSD can be triggered by chemical (e.g. K^+, glutamate), mechanical or electrical stimulation of the cortex [83]. In animals, it was reported that CSD can be also triggered by sensory activation of the brainstem [204]; however, this needs to be further validated. Induction of CSD may be measured using electrophysiological techniques, commonly field potential recordings, and blood flow changes through laser Doppler flowmetry. Optical intrinsic signal imaging has been also used to monitor blood flow/metabolic changes that appear in connection with CSD [200]. In freely moving animals, induction of CSD was shown to induce motor freezing [205], without the development of cutaneous allodynia or any other nociceptive-like behaviour [206, 207].

The CSD model has been widely used to examine the efficacy of different treatments. Although CSD is a "yes or no" event, in the literature, treatments are considered as potentially effective not only when they block induction of CSD but also when the rate of propagation or amplitude of DC shift and blood flow changes are suppressed. A reduction of the sum of CSD waves in the case of K^+-induced CSD and an increase in the threshold of electrical activation of the cortex are also considered as important outcomes of therapeutic efficacy [208]. Drugs identified to have a prophylactic effect on CSD in rats after many weeks of daily treatment are valproate, topiramate, propranolol, amitriptyline and methysergide [209]. Lamotrigine was also shown to block K^+-induced CSD, possibly through interactions with the glutamatergic system [210]. CSD blockade with amiloride, via the acid-sensing ion channel 1a, was found to be attributed to its preventive role in a small open clinical trial [211, 212]. Apart from treatment with medications, cortical neuromodulation by transcranial magnetic stimulation has been found to be effective in both K^+- and mechanically induced CSD [213, 214]. Triptans are not expected to have an effect in CSD or the aura in humans [215], although a role for serotonin in the maintenance of balanced cortical activation has been suggested, given that animals depleted of serotonin demonstrate an enhanced cortical sensitivity to K^+ application [216].

Recent studies in the CSD model have focused on the potential mechanisms through which CSD may interact with the ascending trigeminothalamic pathway, either peripherally through activation of the trigeminovascular system or centrally through cortico-subcortical networks. Elevated Fos levels can be found due to CSD in various areas of the brain and in the TCC [217, 218], although contradictory preclinical data exist for the TCC [219]. Bolay et al. [220] demonstrated that CSD causes vasodilation of meningeal blood vessels, which is accompanied by activation of the TCC, manifested by the presence of Fos-positive cells. Recent electrophysiological studies, combining mechanical, chemical or electrically induced CSD and electrophysiological recordings from trigeminal ganglion or TCC neurons, demonstrated that meningeal nociceptors may be activated following CSD as spontaneous activity of neurons was facilitated 50 % of the time [221]. These outcomes suggest that CSD may induce activation of the ascending trigeminothalamic pathway through sensitisation of peripheral nociceptors, presumably following the CSD-induced release of substances from the cortex that may activate such channels through diffusion in the subarachnoid space, as initially suggested by Bolay et al. [220]. Karatas and colleagues [222] suggested recently that this occurs due to activation of the gap junction protein Pannexin 1, as inhibition of the signalling cascade activated by neuronal Pannexin 1 abolished CSD-induced trigeminovascular activation and dural mast cell degranulation [222]. The role of Pannexin 1 and gap junction molecules in migraine, however, remains to be determined. The gap junction channel modulator tonabersat (SB-220453) was shown to be decreasing the number of CSD waves induced by K^+ [223]; however, its efficacy in the clinic is questioned [224]. Other studies have shown that CSD can alter positively or negatively the activity of second-order neurons, without interactions with peripheral inputs, or the spread of CSD to subcortical areas in an otherwise healthy brain. This could be achieved through activation of corticospinal pathways, depending on the site of cortical stimulation [225], or indirectly through cortico-brainstem pathways [226, 227]. Andreou et al. have also shown that CSD may sensitise the ipsilateral sensory thalamic nuclei VPM and Po, through direct corticothalamic pathway activation [228, 229]. Whether these changes are sufficient to elicit the perception of migraine headache in patients is unclear.

Although to date the mechanisms and interactions of CSD with pain pathways have not been fully understood, recent advances shed some light on possible interactive mechanisms and provide important information about the phenomenon itself. The model serves well the scientific experimentation for the identification of new drugs specific for migraine aura; however, it remains to be established if agents that prevent the aura symptoms may also treat the migraine headache or even prevent a migraine attack from being triggered.

2.6 Genetic Models of Migraine

To date, genetic models of migraine include genetically modified mice in which known human mutations of familial hemiplegic migraine (FHM) and of familial advanced sleep phase (FASP) syndrome have been knocked in their genome. The

genome-wide association studies (GWAS) performed to date have identified a number of genes that may be associated with more common forms of migraine. These studies might offer future opportunities for the development of further genetic models of migraine that could shed light on migraine pathophysiology and treatment development.

2.6.1 Familial Hemiplegic Migraine Models

Identification of autosomal-dominant gene mutations in familial hemiplegic migraine (FHM) patients, a rare subtype of migraine with prominent aura symptoms, allowed for the first time the development of genetic models of migraine [230]. FHM1 mutations affect the CACNA1A gene, FHM2 mutations affect the ATP1A2 gene and FHM3 mutations affect the SCN1A gene. So far, there have been no transgenic mice carrying the human FHM3 mutations. In contrast, knock-in models utilising two CACNA1A mutations of FHM1, the R192Q and S218L, have been developed, as well as one model of FHM2, carrying the human W887R mutation [85, 231]. These animal models have been used in conjunction with a number of assays described above, ranging from immunohistochemistry to behavioural tests, in a number of studies that aimed to gain insight into the pathophysiology of FHM and of more common types of migraine.

CSD experiments demonstrated a decreased threshold for CSD induction in the R192Q and S218L FHM1 mutant mice [232, 233]. Similarly to patients who carry the S218L mutation, S218L FHM1 mutant mice were also found to be predisposed to severe brain oedema [232]. One model of FHM2, carrying the human W887R mutation, has been also developed [231] and, likewise to the FHM1 models, is characterised by a decreased induction threshold of CSD and an increased velocity of propagation of the spreading wave [231]. Overall, both FHM1 and FHM2 genetic models appear to model significantly the clinical FHM phenotype as described in patients, making them excellent tools for further investigations that would aim to develop FHM-specific pharmacological treatment.

Although behavioural tests suggest that these animals demonstrate spontaneous nociceptive-like and photophobia-like behaviour [234, 235], their ability to model common types of migraine has been questioned. FHM shares many phenotypical similarities with common types of migraine, suggesting the existence of common neurobiological pathways. However, despite the well-established importance of CGRP in the pathophysiology of common types of migraine [89], immunohistological identification of CGRP in the TCC of FHM1 mice showed a reduced expression in trigeminal ganglia neurons and TCC [236]. In agreement with this, FHM patients with known mutations in the CACNA1A and ATP1A2 genes do not show hypersensitivity to either CGRP or NO donors' infusion, as characteristically seen in migraine patients [237, 238]. Furthermore, identification of Fos-positive cells in FHM mice following trigeminovascular stimulation demonstrated an unpredicted reduced number of positive profiles in the TCC compared to wild-type animals [239]. The opposite

was also seen in the brainstem and hypothalamic nuclei where one would normally expect a reduced modulatory role [240]. These data indicate that the pathophysiological pathways underlying migraine headache in FHM may be different from the common types of migraine; however, further studies are needed to conclude to such a hypothesis. Nevertheless, given that all identified FHM mutations are highly susceptible to CSD propagation or to excitability of neuronal tissue, FHM genetic models can advance our understanding of migraine aura and its treatment.

2.6.2 Casein Kinase 1δ (CK1δ) Model

More recently, a mutation in the clock gene encoding casein kinase 1δ (T44A), which results in reduced enzymatic activity, has been described in patients with familial advanced sleep phase (FASP) syndrome. This syndrome is characterised by altered circadian rhythms, reflected in early morning waking and early sleep times [241]. In one family, FASP was found to be associated with migraine with and without aura [242], suggesting a functional relation of this mutation with migraine neurobiology. Genetically engineered mice that carry the T44A human gene mutation, similarly to FHM mice, also showed an increased susceptibility to CSD induction, and hypersensitive behavioural responses, associated with increased Fos expression in the TCC following infusion of NTG [242]. These data suggest a potential role of this gene in migraine neurobiology and the prospective use of this genetic model in studies looking into new treatments and the neurobiology involved in migraine. However, this model needs to be further validated in order to conclude if findings from this rare form of migraine may be extrapolated to more common forms of the disorder. The mutation itself needs to be further validated as a migraine mutation in a bigger population of patients.

2.7 Conscious Models of Episodic or Chronic Migraine Pain

A major limitation of the models described earlier is that they do not reflect the repeated episodic nature of migraine attacks and hence the neuronal plasticity may develop in migraine patients over the course of the disease. The need of a model that better represents the episodic nature of migraine attacks has been long stated [243]. Chronic models of migraine are generally developed using similar assays as those described above; however, they employ freely moving awake animals and usually behavioural tests in order to assess the model's phenotype and potential therapeutic outcomes.

Oshinsky and Gomonchareonsiri [243] developed probably the first model of episodic migraine, by inducing trigeminovascular activation through repeated applications of inflammatory soup through a cannula fixed on top of the dura mater of awake behaving rats. The authors demonstrated that over a period of 4 weeks, rats developed increased mechanical cutaneous sensitivity at the periorbital area following

infusion of a NO donor. Using in vivo microdialysis, the authors further showed that these behavioural changes occur in parallel to increased glutamate levels in the TCC, suggesting a state of increased excitatory neurotransmission [243]. Using the same model, Stucky et al. [244] demonstrated pronounced sex differences, with female rats developing behavioural changes at a lower dose of inflammatory agents and for a longer duration. Additionally, using real-time polymerase chain reaction, female rats demonstrated lower mRNA levels of the CGRP receptor subunits. Beyond evoked pain, utilising video recordings and second-by-second analysis, this model was shown to present changes in spontaneous behaviour [245]. In these rats, behavioural observations indicated increased facial grooming ipsilateral to the cannula implantation, provoked following infusion of the inflammatory soup, compared to animals that were infused with saline. Additionally, these animals demonstrated an increased freezing and resting behaviour, which was significantly reduced by zolmitriptan and ketorolac, but not acetaminophen [245]. Similarly to the inflammatory soup repeated application, Dong and colleagues [246] employed electrical stimulation of dural vessels in awake rats that also induced trigeminovascular activation. They demonstrated that high-frequency stimulation can elicit increased facial grooming and head-flick behaviour that is blocked by morphine or rizatriptan. Pradhan and colleagues [247] characterised recently a model of chronic migraine, in which chronic intermitted administration of NO donors resulted in the development of chronic extracranial hyperalgesia, assessed with mechanical stimuli over the plantar surface. Female mice showed a stronger phenotype compared to male. Chronic hyperalgesia was found to be suppressed by sumatriptan, by the migraine preventative topiramate and by different δ-opioid receptor agonists [247, 248].

Finally, Oshinsky et al. [249] isolated a colony of Sprague-Dawley rats that appear to experience episodic trigeminal allodynia. Using von Frey mechanical stimulation of the trigeminal region, Oshinsky et al. characterised "spontaneous trigeminal allodynia" rats as those that have mechanical thresholds in the normal range (8–15 g) on some days and thresholds as low as 1.0 g on other days. Using this behavioural assay, a rat with spontaneous episodic trigeminal sensitivity was discovered and thought to represent a model of spontaneous trigeminal allodynia. Low withdrawal thresholds were also found in the masseter muscle region of the jaw but not in the hind paws. Subsequent mating of these rats showed that the trait is inherited, suggesting a similarity to the inherited nature of migraine in humans. These rats were also shown to have increased sensitivity to sound, similar to phonophobia in migraineurs [250]. Using von Frey filaments, the authors further found an increased sensitivity to the chemical headache triggers NTG and CGRP. Finally, the rats' periorbital mechanical threshold was normalised by clinically proven acute and preventive pharmacological migraine treatments. Sumatriptan, ketorolac and dihydroergotamine DHE transiently returned the periorbital nociceptive thresholds of the spontaneous trigeminal allodynia rats to normal levels. The migraine preventive treatment valproic acid restored the periorbital pain threshold to normal levels during treatment. Since the "spontaneous trigeminal allodynia" shares many of these phenotypes and sensitivities of migraineurs, the authors suggest that they can be a good model for studying the pathophysiology and drug discovery for migraine.

The main limitation of these models, despite their usefulness in exploring episodic trigeminovascular activation, lies on the interpretation of animal behaviour and its translation to human pain. Measuring hypersensitivity in rodents can be challenging and requires expert training and good laboratory practices, particularly for assessing hypersensitivity in the craniofacial region [251]. Indeed, animal studies of migraine have predominantly concentrated on modelling trigeminovascular nociception in the anaesthetised animal; as such, many of the important neurological features that accompany migraine are overlooked [83]. However, proof of concept for the effectiveness of these models needs to be provided by other laboratories, and this needs to include evaluation of the models using nonclinically active treatments. Adaptation of the models with assays that demonstrate activation of the trigeminothalamic pathway will be an advantage in the field of conscious migraine behavioural research.

2.8 Medication Overuse: Latent Sensitisation Model

Developed in the Porecca's lab, the latent sensitisation animal model aimed to represent the medication overuse headache, which is often developed in migraine patients following chronic administration of triptans and other acute painkillers [1]. The prevalence of cutaneous allodynia in MOH patients is higher than in episodic migraine sufferers [252]. Central sensitisation is thought to be the underlying mechanism for the development of a chronic migraineurs status.

In the latent sensitisation model, rats that were exposed to chronic administration of triptans [253, 254] or morphine [255] developed behavioural signs of cutaneous allodynia. Additionally, these animals were more sensitive to NO donors' infusion and demonstrated further signs of central sensitisation following exposure to stress stimuli [253–256]. These behavioural changes were accompanied by increased expression of CGRP and nNOS in the trigeminal ganglia and TCC [253–255]. Importantly, these changes persisted over a prolonged period of time following the cease of chronic drug administration. This long-lasting state of hypersensitivity to different stimuli was considered to reveal a state of "latent sensitisation" [256]. More recent data from the same laboratory also suggest that rats exposed to chronic sumatriptan administration have lower threshold of CSD induction compared to animals that received chronic administration of saline that could be reversed by topiramate administration [257].

Although the development of a medication overuse model in migraine is certainly desirable, particularly for testing the mechanisms of chronic migraine, the latent sensitisation model needs to be validated by other laboratories as well. More importantly, the efficacy of the model needs to be tested against treatments that are not known to induce MOH, such as migraine preventives that are taken on a daily basis, and even against chronic exposure to non-pain-related treatments. Additionally, given reports on the rather unlikely central action of sumatriptan due to its inability to cross the BBB, further clarifications are needed as to why this drug may interfere

with the induction properties of CSD. Nevertheless, the model has yet a lot to offer, particularly in terms of neuronal plasticity that most likely underlies the development of medication overuse altered phenotype.

2.9 Conclusions

Preclinical investigations in migraine research involve animal models that have been developed over the years to better model our current understanding of disease mechanisms. Modelling of migraine in animal models has been based on clinical evidence coming from migraineurs. While the effectiveness of some of the newer described models is still pending, the field is blessed with well-established models that reliably replicate aspects of the migraine phenotype and screen satisfactorily potential therapeutics. In particular, animal models of migraine that involve activation of the trigeminovascular system, and CSD, the migraine aura model, are considered well-reliable pharmacological tools. One of the biggest challenges in developing a suitable animal model for the study of migraine is the extent of clinical symptoms required to be present in order to fulfil the diagnosis criteria [1]. Therefore, an ideal animal model for the study of migraine should resemble as close as possible this multi-symptom complexity in the form of quantifiable correlates [83]. The need for animal models which have aspects of migraine symptoms other than the pain is thus urgent. Future studies should aim to model these features along with the use of cutting edge techniques such as optogenetics, with the scope of facilitating our understanding of migraine, and to better replicate brain activation as seen in patients. Given the ongoing GWAS in migraine, the development of genetically modified animal models that will replicate the phenotype of common forms of migraine should be anticipated in the years to come.

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