The Neural Mechanisms of Sleep and Migraine

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The Neural Mechanisms of Sleep and Migraine
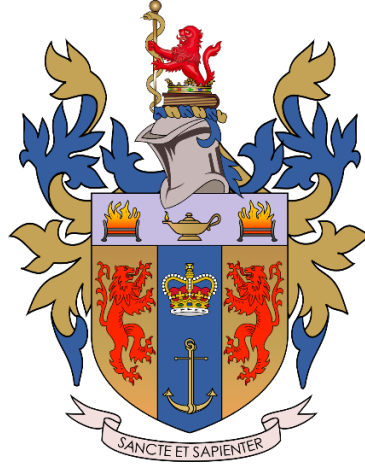
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Declaration

I, Emily Charlotte Stanyer confirm that the work in this thesis is my own. The experimental work contained herein is my own except where explicitly stated otherwise. This work has not been submitted for any other degree or professional qualification. The copyright of this thesis rests with the author and no quotation from it or information derived from it may be published without proper acknowledgement.

Abstract

Whilst a bidirectional relationship between sleep and migraine has long been postulated, this remains mainly speculative, and the underlying neural mechanisms remain to be determined. In this thesis we sought to explore this with clinical and preclinical methodologies. It was hypothesised that disrupted sleep-wake and nociception-regulating neural networks including key brainstem and diencephalic structures alter the thresholds for attack initiation and increase migraine susceptibility.

Firstly, we used a meta-analytic approach to determine whether migraine patients have altered sleep, identifying that they have poorer subjective sleep quality and altered sleep physiology including reduced rapid-eye-movement sleep, compared to healthy controls. By collating data from users of the Migraine Buddy application (Healint Ltd.) and conducting Bayesian regression models we explored whether changes in sleep were predictors of an attack and conversely whether experiencing an attack would predict changes in subsequent sleep. We determined that interrupted sleep and deviations from typical sleep were potential predictors of a next day migraine attack but having an attack did not predict sleep duration.

Secondly, we utilised mouse models of sleep deprivation and demonstrated that this led to orofacial mechanical allodynia - a commonly reported migraine phenotype indicative of sensitisation of the trigeminovascular system. Mechanistic insight was provided in that orexin-A, a hypothalamic arousal-promoting peptide which stabilises sleep-wake transitions reversed this phenotype.

Finally, we explored whether familial natural short sleepers (FNSS) which are reported to have increased orexin expression, are less susceptible to migraine-related

phenotypes using a transgenic mouse line harbouring the P384R mutation in the *h*DEC2 gene. We observed no significant differences in migraine-related phenotypes at baseline, however, when exposed to a clinical migraine trigger (nitroglycerin) FNSS mice demonstrated reduced orofacial hypersensitivity and photophobia, indicative of decreased migraine susceptibility. FNSS also displayed alterations in metabolites underlying energy metabolism and oxidative stress, suggesting a potential link between metabolism and headache pathophysiology.

Taken together, the data in this thesis has shed light on the relationship between sleep and migraine, highlighting alterations in sleep as a potential precipitant of migraine attacks, and identifying genetic mechanisms underlying sleep regulation which may curtail migraine development, as well as possible therapeutic targets based on the orexinergic system. Although further work is needed to fully understand this neural basis, this has promising clinical implications and has furthered our understanding of migraine pathophysiology.

Covid-19 Impact Statement:

The COVID-19 pandemic had a significant impact on this PhD project. Significant proportions of the planned preclinical work – including the electrophysiology and electroencephalography (EEG) surgery had to be paused for six months as close-contact training was not permitted. Moreover, when training was allowed to resume, numbers in the laboratory were extremely limited and there were substantial delays with deliveries of essential consumables.

Whilst efforts were taken to mitigate the impact by performing meta-analyses and analysis of data taken from the Migraine Buddy application, there were remaining experiments to be performed. This included EEG recordings on the natural short sleeper transgenic mouse line and immunohistochemistry for orexin peptides and receptors on sleep deprivation tissue.

Acknowledgements

Publications

Related publications:

Stanyer, E. C., Creeney, H., Nesbitt, A. D., Holland, P. R., & Hoffmann, J. (2021). Subjective Sleep Quality and Sleep Architecture in Patients With Migraine: A Meta-analysis. *Neurology*, 97(16), e1620-e1631.

Stanyer, E.C., Brookes, J., Pang, J.R. et al. Investigating the relationship between sleep and migraine in a global sample: a Bayesian cross-sectional approach. *The Journal of Headache and Pain* 24, 123 (2023).

Stanyer, E.C., Hoffmann J., Holland P. R. (2022) Orexin-A recovers sleep-deprivation induced periorbital allodynia in a preclinical migraine model. *Cephalalgia* (Abstract*)*

Stanyer, E.C., Hoffmann, J., & Holland, P. R. (2022). The impact of acute and chronic sleep deprivation on migraine-related phenotypes. *Journal of Sleep Research* (Vol. 31; Abstract)

Unrelated publications:

Al-Hassany, L., Boucherie, D. M., Creeney, H., van Drie, R. W., Farham, F., **Stanyer E.C**...& European Headache Federation School of Advanced Studies (EHF-SAS). (2023). Future targets for migraine treatment beyond CGRP. *The Journal of Headache and Pain*, 24(1), 76.

Labastida-Ramirez. A., Caronna E., Gollion C., **Stanyer E.C**...(2023) Site and Mode of Action of Therapies Targeting CGRP Signalling. *The Journal of Headache and Pain, 24(1), 125.*

Onofri, A., Pensato, U., Rosignoli, C., Wells-Gatnik, W., **Stanyer, E.C**, Ornello, R...(2023). Primary headache epidemiology in children and adolescents: a systematic review and meta-analysis. *The Journal of Headache and Pain*, 24(1), 8.

Stanyer, E. C., Baniqued, P. D. E., Awais, M., Kouara, L., Davies, A. G., Killan, E. C., & Mushtaq, F. (2022). The impact of acoustic stimulation during sleep on memory and sleep architecture: A meta-analysis. *Journal of Sleep Research,* 31(3), e13385.

Table of Contents

List of Tables

List of Figures

Abbreviations

5-HT	5-hydroxytryptamine
BAC	Bacterial artificial chromosome
BBB	Blood-brain barrier
BOLD	Blood Oxygenation Level Dependent
BSU	Biological Services Unit
ACC	Anterior cingulate cortex
ALT	Alanine aminotransferase
ARAS	Ascending reticular activating system
ADP	Adenosine Diphosphate
ATP	Adenosine Triphosphate
CAP	Cyclic alternating pattern
CGRP	Calcitonin gene-related peptide
CK1δ	Casein kinase 1 delta
CK1ε	Casein kinase 1 epsilon
CNS	Central nervous system
CSD	Cortical spreading depression
CSF	Cerebrospinal fluid
DEC2	Differentiated Embryo Chondrocyte 2
DAB	3,3′-Diaminobenzidine
DAPI	4′,6-diamidino-2-phenylindole
DMR	Dorsal median raphe nucleus
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EEG	Electroencephalography
FASP	Familial advanced sleep phase
FHM	Familial hemiplegic migraine
FNSS	Familial natural short sleepers
GABA	Gamma-Aminobutyric acid
gDNA	Genomic DNA
GPCR	G-Protein Coupled Receptor
LC	Locus coeruleus
LDT	Lateral dorsal tegmentum
LH	Lateral hypothalamus
MA	Migraine with aura
MCH	Melanin concentrating hormone
MIDAS	Migraine Disability Assessment Test
MoA	Migraine without aura
NAD+	Nicotinamide adenine dinucleotide
NAM	Nicotinamide
NDS	Normal donkey serum
NREM	Non-rapid-eye-movement sleep

OX₁R	Orexin-1 receptor
OX₂R	Orexin-2 receptor
OXA	Orexin-A
OXB	Orexin-B
NSRM	Non-sleep-related migraine
NTG	Nitroglycerin
PACAP	Pituitary adenylate cyclase-activating peptide
PAG	Periaqueductal gray
PCr	Phosphocreatine
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
PPT	Pedunculopontine tegmentum
PSD	Power spectral density
PSG	Polysomnography
PSQI	Pittsburgh Sleep Quality Index
qPCR	Quantitative polymerase chain reaction
RBD	Rapid-eye-movement sleep behaviour disorder
REM	Rapid-eye-movement sleep
ROI	Region of interest
RPM	Rotations per minute
RVM	Rostral ventral medulla
SEM	Standard error of the mean
SCN	Suprachiasmatic nucleus of the hypothalamus
SOREMP	Sleep-onset REM period
SD	Standard deviation
SRM	Sleep-related migraine
SWS	Slow-wave sleep
TCC	Trigeminal cervical complex
TG	Trigeminal ganglion
TMN	Tuberomammillary nucleus
TNC	Trigeminal nucleus caudalis
TST	Total sleep time
VLPO	Ventrolateral preoptic nucleus

Chapter 1: Introduction

'If sleep does not serve an absolutely vital function, then it is the biggest mistake the

evolutionary process has ever made' - Allan Rechtschaffen

1.1   Migraine

Primary headache disorders refer to headache disorders which lack a causative pathology such as trauma or disease (Benoliel & Eliav, 2013). This includes but is not limited to: migraine, cluster headache, and tension-type headache (The International Classification of Headache Disorders, 3rd edition, 2018). Primary headaches in general place a large toll on health resources leading to direct and indirect costs of €112 billion across Europe per annum (Linde et al., 2012).

One of the most debilitating of the primary headache disorders is migraine, which is the second leading cause of disability worldwide (Vos et al., 2017). Migraine impacts more than one billion people, and creates a significant socioeconomic burden through direct and indirect costs (Lanteri-Minet, 2013). For example, migraine is estimated to cost the United States $19.6 billion (Stewart et al., 2003) and the European Union €18.5 billion annually (Gustavsson et al., 2011), and is amongst the most burdensome yet under-researched medical conditions (Directorate-General for Research and Innovation (European Commission) et al., 2023). Despite this burden, and advances in treatment, there remains a major need to develop new targeted therapies with improved efficacy and reduced side effects, and to develop a greater understanding of migraine pathophysiology.

1.1.1 Epidemiology of Migraine

Whilst the 1-year prevalence of migraine is 14-15%, prevalence varies according to sex, age, cultural, and geographic factors (Steiner & Stovner, 2023). For example, migraine is more common in women with 18% being afflicted versus 6% of men (Buse, Loder, et al., 2013; Lagman-Bartolome & Lay, 2019), with the maximal sex difference occurring between the age of 30 and 45 (Vetvik, 2019). The prevalence of migraine changes across the lifespan; attacks typically commence in childhood and increase

with age, peaking between 30 and 39 years of age before declining, particularly after the onset of menopause in women (Lyngberg et al., 2005; Ripa et al., 2015). Pre-puberty however, there is no overt difference in migraine incidence between males and females (Onofri et al., 2023), suggesting a role of sex hormones in migraine pathophysiology (Pakalnis, 2016).

Infrequent or occasional migraine attacks are termed episodic migraine. Whereas migraine is classified as chronic when a patient experiences >15 headache days per month out of which at least eight fulfil the diagnostic criteria for migraine (The International Classification of Headache Disorders, 3rd Edition, 2018). Approximately 3% of migraine patients will progress to a chronic state each year (Lipton, 2009) and the prevalence of chronic migraine has a tendency to increase with ageing, peaking at age 40 (Burstein et al., 2015; Buse et al., 2012).

1.1.2 Phases of Migraine

Migraine is most commonly characterised by recurrent unilateral throbbing pain with moderate to severe intensity in the periorbital region which can last from 4-72 hours. Migraine can be further subdivided into migraine with aura (MA) and migraine without aura (MoA). Aura is a transient, reversible feature which precedes or accompanies the migraine headache phase in around 20-30% of patients (Bigal et al., 2006; see section 1.1.3). See **Table 1** for the diagnostic criteria for MA and MoA.

Migraine can be divided into four phases: the premonitory phase (or prodrome), the aura phase, the headache phase, and the postdrome phase (Goadsby, 2005; see **Figure 1**). However, there is extreme intra and inter-individual variation and not all patients will experience all phases, and not every attack will assume these phases (Lai & Dilli, 2020). In the days and hours leading up to a migraine attack, around 84% of patients commonly report premonitory symptoms such as mood changes, food

cravings, abnormal levels of tiredness, polyuria, cognitive difficulties, and frequent yawning (Gago-Veiga et al., 2019; Quintela et al., 2006). Then the headache phase occurs, during which some patients additionally experience aura (see section 1.1.3). Patients often experience other symptoms during the headache phase including: nausea, fatigue, photophobia (sensitivity to light), phonophobia (sensitivity to sound), osmophobia (sensitivity to smell), frequent yawning, and variable levels of cranial autonomic symptoms (Goadsby & Holland, 2019; Obermann et al., 2007; see **Table 2**). After the throbbing, often unilateral head pain has subsided, the postdrome phase begins. Sixty-seven percent of migraine patients report a postdrome phase (Giffin et al., 2005), which can last on average 25.2 hours after cessation of the headache phase (Kelman, 2006). Common symptoms during this phase include cognitive difficulties, abnormal levels of tiredness, dizziness, and mood swings (Bose & Goadsby, 2016).
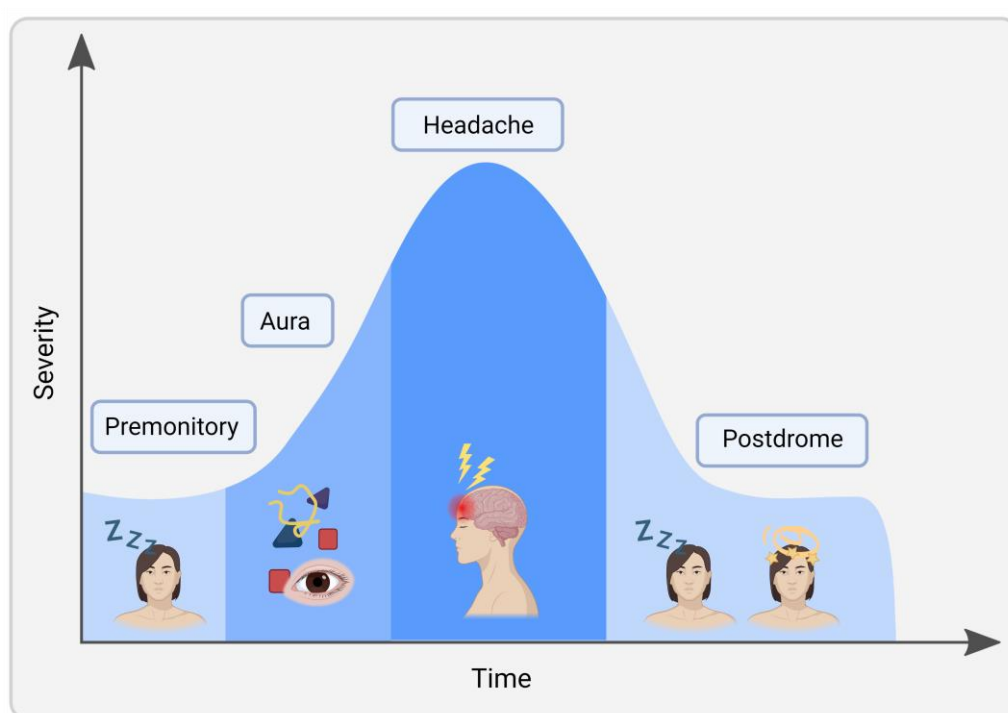
Figure 1: Phases of migraine across time and their severity
Migraine attacks commonly begin with the premonitory phase with symptoms including frequent yawning, fatigue, nausea, and thirst. Some patients also experience aura in which they experience neurological disturbances, before the often accompanying or subsequent migraine attack phase which is characterised by severe unilateral pain in the periorbital region. The postdrome follows in which patients experience many symptoms including cognitive and mood disturbances and fatigue.

Table 1: Diagnostic criteria for migraine with aura (MA) and migraine without aura (MoA) as defined by ICHD-III

Migraine without aura (MoA)

A. At least five attacks fulfilling criteria B-D

B. Headache attacks lasting 4-72 hours (untreated or unsuccessfully treated)

C. Headache has at least two of the following four characteristics:

    1. Unilateral location

    2. Pulsating quality

    3. Moderate or severe pain intensity

    4. Aggravation by or causing avoidance of routine physical activity (e.g. walking or climbing stairs

D. During headache at least one of the following:

    1. Nausea and/or vomiting

    2. Photophobia and phonophobia

E. Not better accounted for by another ICHD-III diagnosis

Migraine with aura (MA)

A. At least two attacks fulfilling criteria B and C

B. One or more of the following fully reversible aura symptoms:

    1. visual

    2. sensory

    3. speech and/or language

    4. motor

    5. brainstem

    6. retinal

C. At least two of the following four characteristics:

    1. at least one aura symptom spreads gradually over ≥5 minutes, and/or two or more symptoms occur in succession

    2. each individual aura symptom lasts 5-60 minutes

    3. at least one aura symptom is unilateral

    4. the aura is accompanied, or followed within 60 minutes, by headache

D. Not better accounted for by another ICHD-III diagnosis, and transient ischaemic attack has been excluded

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Table 2: Symptoms associated with migraine at each phase

Symptom	Premonitory	Aura	Headache	Postdrome
Fatigue	✓		✓	✓
Frequent yawning	✓			✓
Mood changes	✓			✓
Unilateral throbbing pain			✓	
Motor problems		✓		
Visual disturbance		✓		
Language/speech problems		✓		
Cutaneous allodynia			✓	
Cognitive difficulties	✓			✓
Nausea and/or vomiting			✓	
Osmophobia			✓	
Photophobia			✓	
Phonophobia			✓	
Food cravings	✓			
Stiff neck	✓			✓
Dizziness				✓
Frequent urination				✓
Thirst				✓
Digestive symptoms				✓

1.1.3 Aura

Aura is a focal, reversible symptom which is most often visual in nature and is typically reported as kaleidoscopic visual phenomena including flashing lights, colours, and geometric patterns (Cutrer & Huerter, 2007). Whilst it is primarily associated with migraine, aura may also accompany other headache disorders or occur independently (Goadsby, 2001). 98-99% of patients with MA report a visual aura

(Russell & Olesen, 1996; Viana et al., 2019), whereas 36% report a sensory and 10% report a language aura (Viana et al., 2017). Visual aura can present as either positive (lights, patterns) or negative (scotoma) features. It tends to occur gradually, and each individual aura symptom may last for 5-60 minutes, with a median duration of 20-30 minutes. Some patients will progress through different sensory modalities in succession, for example experiencing a visual followed by a sensory and then a language/speech aura (DeLange & Cutrer, 2014). In a small number of cases (<5%), no headache follows or accompanies the aura (Russell et al., 1995). Conversely, a small number of patients (~9%) experience aura after the headache pain has resolved (Viana et al., 2017). At present, it is unclear why some migraines occur with aura and others do not (Charles, 2018), thus there is high inter and intra-variability in MA. What's more, when migraines are experimentally triggered using compounds (e.g. nitroglycerin) in patients with MA, this induces headache but does not induce aura, suggesting a potential differential mechanism for MA initiation versus MoA (Christiansen et al., 1999). That being said, preventive migraine therapies reduce aura-related mechanisms in animal models (see section 1.1.4 below), suggesting a common pathophysiology underlying MA and MoA (Takizawa et al., 2020).

1.1.4 Cortical Spreading Depression

The underlying mechanism of aura is thought to be cortical spreading depression (CSD). Originally observed in the 1940s by Karl Lashley (Lashley, 1941) and then further investigated by Aristides Leão (Leao, 1944), CSD refers to a transient wave of neuronal and glial depolarisation which propagates across the cortex at a rate of 3-5mm/minute, followed by a prolonged suppression of cortical activity (~30 minutes) initially observed in animal models. Focal hyperemia also precedes the wave of neuronal depolarisation (Sakai & Meyer, 1979).

At the molecular level, CSD is thought to be due to alterations in transmembrane ion gradients, resulting in large influxes of ions such as calcium (Ca), sodium (Na), potassium (K), and water (Canals et al., 2005; Somjen, 2001). Functional magnetic resonance imaging (fMRI) studies have demonstrated neural activity in the visual cortex in human migraine patients which maps onto the characteristics of CSD found in animal models (Hadjikhani et al., 2001). CSD is postulated to be the physiological correlate of aura as the speed of the wave corresponds to the speed at which aura symptoms progress (Charles & Baca, 2013). Whilst this is currently contested, it is suggested that CSD may trigger the initiation of migraine attacks as it has been shown to activate areas involved in trigeminal nociception such as the trigeminal nucleus caudalis (TNC; Moskowitz et al., 1993; Zhang et al., 2011), and CSD leads to vasodilation and release of inflammatory neuropeptides (Goadsby et al., 1990).

Research has linked CSD susceptibility to genetics. For example, genetic models of migraine such as the familial hemiplegic migraine (FHM) mouse show increased susceptibility to CSD events (Hansen, 2010). Furthermore, a gene involved in regulating the circadian clock was implicated for the first time in migraine pathophysiology and cortical excitability. For example, a loss of function mutation in the gene casein kinase Iδ that causes circadian rhythm disruption resulted in a very high penetrance of migraine with aura in humans and increased susceptibility to CSD events in a mouse model (Brennan et al., 2013).

1.1.5 Cutaneous Allodynia and Central Sensitisation

As well as the throbbing head pain, patients commonly experience sensitivity to touch during attacks. Cutaneous allodynia refers to pain or unpleasantness resulting from an innocuous stimulus such as cold, heat, or pressure. In migraine patients, this often manifests as discomfort when brushing hair, shaving, wearing contact lenses, or

tight clothing (Landy et al., 2004). Studies suggest that up to 79% of patients experience allodynia, and this typically occurs ipsilateral to the headache site (Bigal et al., 2008; Burstein et al., 2010; Guy et al., 2009; Lipton et al., 2008). However, the prevalence of allodynia may be higher due to difficulties in diagnosing allodynia using questionnaires (Mathew et al., 2016). Although, this may be improved when combined with objective methods (Ashkenazi et al., 2007). One-fifth of migraine patients experience severe allodynia (Bigal et al., 2008), and there is a greater prevalence of allodynia in MA and chronic migraine (Lovati et al., 2007).

Allodynia is usually cephalic (87%), but around 18% of patients also experience extracephalic allodynia (Mathew et al., 2004). The modality of allodynia can differ based on the location, as cephalic allodynia is typically mechanical in nature whereas extracephalic is thermal (Guy et al., 2009). The severity and extent of allodynia significantly correlates with headache intensity (Guy et al., 2009; Han et al., 2021); and is thought to be a predictor of response to pharmacological treatment (Burstein et al., 2004), and chronification of migraine (Benatto et al., 2017). This highlights that allodynia is a key feature of migraine which has the added advantage of being readily measurable in preclinical and clinical models (Harriott, Strother, et al., 2019).

Cephalic mechanical allodynia is thought to be due to peripheral and central sensitisation of trigeminal sensory neurons, second order relay neurons in the trigeminal cervical complex (TCC) and their third order targets in the somatosensory thalamus, meaning increased spontaneous firing, evoked activation or enlarged receptive fields to an unchanged stimulus (Landy et al., 2004; Nijs et al., 2019). For a more detailed discussion of this pathway see section 1.2.1. Evidence for this comes from studies where brief chemical irritation of the dura (by applying inflammatory soup) causes trigeminal neurons to be hyperexcitable to mechanical stimuli which

previously evoked little or no response (Strassman et al., 1996). Whereas extracephalic allodynia is thought to be due to sensitisation of third-order trigeminovascular neurons in thalamic nuclei (Akerman et al., 2011; Burstein et al., 2010).

1.2 Pathophysiology of Migraine

Given the array of associated symptoms and phases, migraine is a complex disorder and has an accordingly intricate pathophysiology. There are multiple systems involved at cortical, brainstem, spinal, and diencephalic levels, and therefore migraine may be best viewed as a 'network disorder' rather than arising from a specific set of brain regions (Goadsby & Holland, 2019).

Initial theories of migraine pathophysiology such as the vascular theory proposed that migraine was due solely to dilation of cranial vessels (Wolff, 1948). However, vasodilation of blood vessels by itself is not necessary nor sufficient for initiation of migraine attacks (Goadsby, 2009; Petersen et al., 2005), and blood flow can be normal or even reduced during attacks (Olesen et al., 1990). Moreover, given the symptoms associated with migraine which are non-nociceptive and sensory in nature, recent theories posit a neural origin with a vascular component (Goadsby, Holland, et al., 2017a). Pharmacological treatments for migraine work effectively in many patients and their mechanisms are often based solely on neuronal transmission (Kaube et al., 1993; see section 1.2.5).

Neurological theories therefore focus on the idea that migraine may arise due to abnormal neuronal firing or neurotransmitter release (Arulmozhi et al., 2005). In a similar vein, migraine was theorised to be a disorder of neuronal excitability, akin to epilepsy, in which certain events could 'tip' the brain towards CSD which would trigger attacks (Scheffer et al., 2013; Tottene et al., 2009). Currently the neurovascular theory dominates which suggests that migraine arises from a combination of neurological and

vascular events (Haanes & Edvinsson, 2019; Hoffmann et al., 2019; Tajti et al., 2011). For example, the activation of nerve fibres containing vasoactive neuropeptides which then innervate the cranial blood vessels. These specific pathways will be explored in the following section.

1.2.1 Anatomy of Head Pain

The pain system involves both ascending and descending pathways (Cross, 1994) and migraine could result from both decreased descending inhibition or increased ascending facilitation of nociception. The ascending pathways (section 1.2.2) convey information from peripheral nociceptors via the dorsal horn of the spinal cord to higher levels of the central nervous system (CNS), whereas descending pathways (section 1.2.3) originate in the cortex, thalamus, and brain stem and modulate pain at the spinal level (Stamford, 1995).

1.2.2 Ascending Pathways

It is currently accepted that the headache associated with migraine results from activation and sensitisation of the trigeminovascular pain pathway, as well as brainstem and diencephalic nuclei (Levy, 2010; Noseda & Burstein, 2013; Olesen et al., 2009), with the hypothalamus playing a key role in attack initiation. Evidence for this comes from studies where stimulation of trigeminal afferents leads to migraine-like pain in healthy adults (Ray & Wolff, 1940), increases in extracerebral blood flow (Goadsby & Duckworth, 1987), and release of calcitonin gene-related peptide (CGRP), pituitary adenylate cyclase-activating peptide (PACAP), and substance P (Buzzi et al., 1991; Goadsby et al., 1988, 1990; Samsam et al., 1999).

Pseudo-unipolar trigeminal sensory afferent nociceptors originate in the trigeminal ganglion (TG) of the trigeminal nerve. They innervate intra and extra-cranial tissues such as the meninges, dura mater, as well as the spinal cord TCC

(Pietrobon & Moskowitz, 2013; see **Figure 2**) via the ophthalmic (V1) division of the trigeminal nerve. These nociceptor axons contain vasoactive neuropeptides including CGRP, PACAP, and substance P (Cuello et al., 1978; Edvinsson et al., 2018; Iyengar et al., 2019). The release of these substances leads to inflammation and dilation of blood vessels (Samsam et al., 2010), although the latter is thought to be independent of their migraine-related functions.

These sensory nerve endings project to and terminate in the TCC which comprises the C1 and C2 upper cervical level dorsal horns of the spinal cord and the TNC. These afferent peripheral fibres include the C-fibres, whose terminals are in superficial layers (Rexed lamina: I, II, V, & X) and the A-δ fibres which terminate in deeper layers (Rexed lamina I, II, & V). Aδ fibres are slow, thin, myelinated fibres associated with sharp, pricking, and stinging pain. C fibres are very slow, thin, unmyelinated fibres that are associated with diffuse, thermal, mechanical, and chemical pain (Stein et al., 2020). There is also a reflex connection from the TCC to the parasympathetic system via the superior salivatory nucleus (SuS) and sphenopalatine ganglion (SPG), which is thought to result in any associated cranial autonomic symptoms (Akerman et al., 2012; Holland & Afridi, 2014).

Second-order neurons in the TCC then project rostrally to higher structures such as the rostral ventromedial medulla (RVM), hypothalamus, thalamus, periaqueductal gray (PAG), and locus coeruleus (LC). Third order thalamocortical neurons then synapse on widespread regions of the cortex (Noseda & Burstein, 2013) including different primary and higher order sensory nuclei (e.g. motor: M1, M2 and sensory: S1, S2), as well as other regions including the anterior cingulate cortex (ACC), insula, and prefrontal cortex, which are all involved in nociceptive processing and integration of sensory, cognitive, and affective responses to pain (Singh et al., 2020). Both sensory

and affective pain pathways combine to form the overall experience of pain. The non-pain symptoms accompanying migraine (e.g nausea, photophobia) are thought to be generated by sensitisation of neurons along the pathway, such as in the TCC and the thalamus (Ferrari et al., 2015).
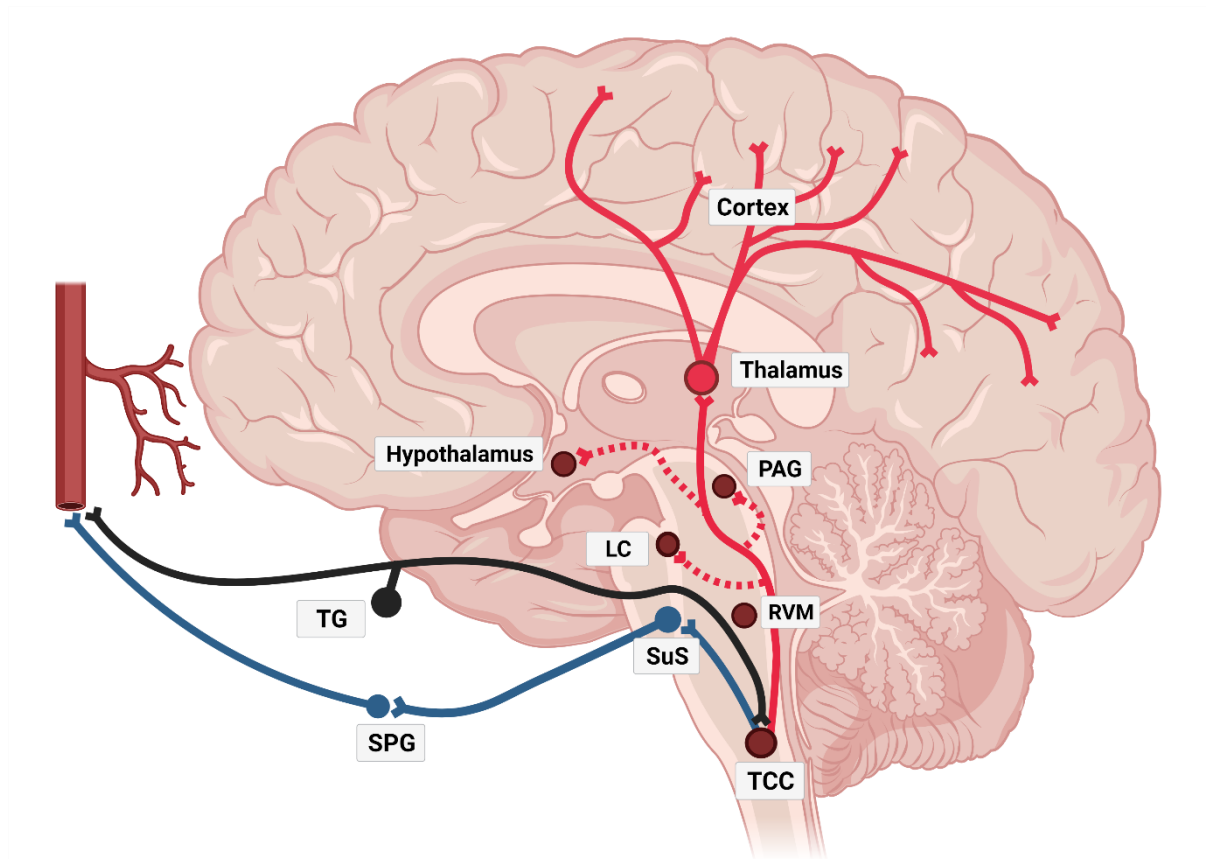
Figure 2: Ascending pain pathways involved in migraine pathophysiology.
Adapted from Goadsby et al. (2017a). Trigeminal sensory afferent nociceptors originate in the trigeminal ganglion (TG) of the trigeminal nerve and innervate intra and extra-cranial tissues such as the meninges and dura mater. These also project to the trigeminocervical complex (TCC) which comprises the C1 and C2 upper cervical level dorsal horns of the spinal cord and the trigeminal nucleus caudalis. There is also a reflex connection from the TCC to the parasympathetic system via the superior salivatory nucleus (SuS) and sphenopalatine ganglion (SPG). Second-order neurons in the TCC then project rostrally to higher cortical structures such as the rostral ventromedial medulla (RVM), hypothalamus, thalamus, periaqueductal gray (PAG), and locus coeruleus (LC). Third order thalamocortical neurons then synapse on widespread regions of the cortex including the primary and secondary motor, somatosensory, and visual cortices.

1.2.3 Descending Pathways

The descending pain modulatory system regulates nociceptive processing in the dorsal horn of the spinal cord in order to determine how pain is ultimately perceived (Denk et al., 2014). In addition to ascending connections, the TCC also receives descending projections from the brain stem and hypothalamic nociceptive nuclei (Akerman et al., 2011). This descending pathway is thus comprised of multiple brain regions which can regulate TCC nociceptive activity (**Figure 3**). This bidirectional control of nociception allows for inhibition or facilitation of nociceptive signals. The brainstem component includes the LC, PAG and RVM. The descending pathway also includes the amygdala and the ACC. These regions interact with nociceptive processing to contribute to the affective aspects of pain (Eippert et al., 2009).

Evidence for a role of descending modulation in migraine comes from functional imaging and preclinical studies. Alterations in the descending pain modulatory pathway are apparent prior to initiation of a migraine attack (Mungoven et al., 2022), as well as interictally (Moulton et al., 2008; Schwedt et al., 2014). For example, Maniyar et al. (2014) used positron emission tomography and found activation of the PAG and LC during nitroglycerin-triggered migraine attacks. Interestingly, these activations were localised ipsilateral to the headache side as has been reported in other studies (Afridi, Matharu, et al., 2005), suggesting that the activation of the brainstem before headache onset may be involved in headache generation.
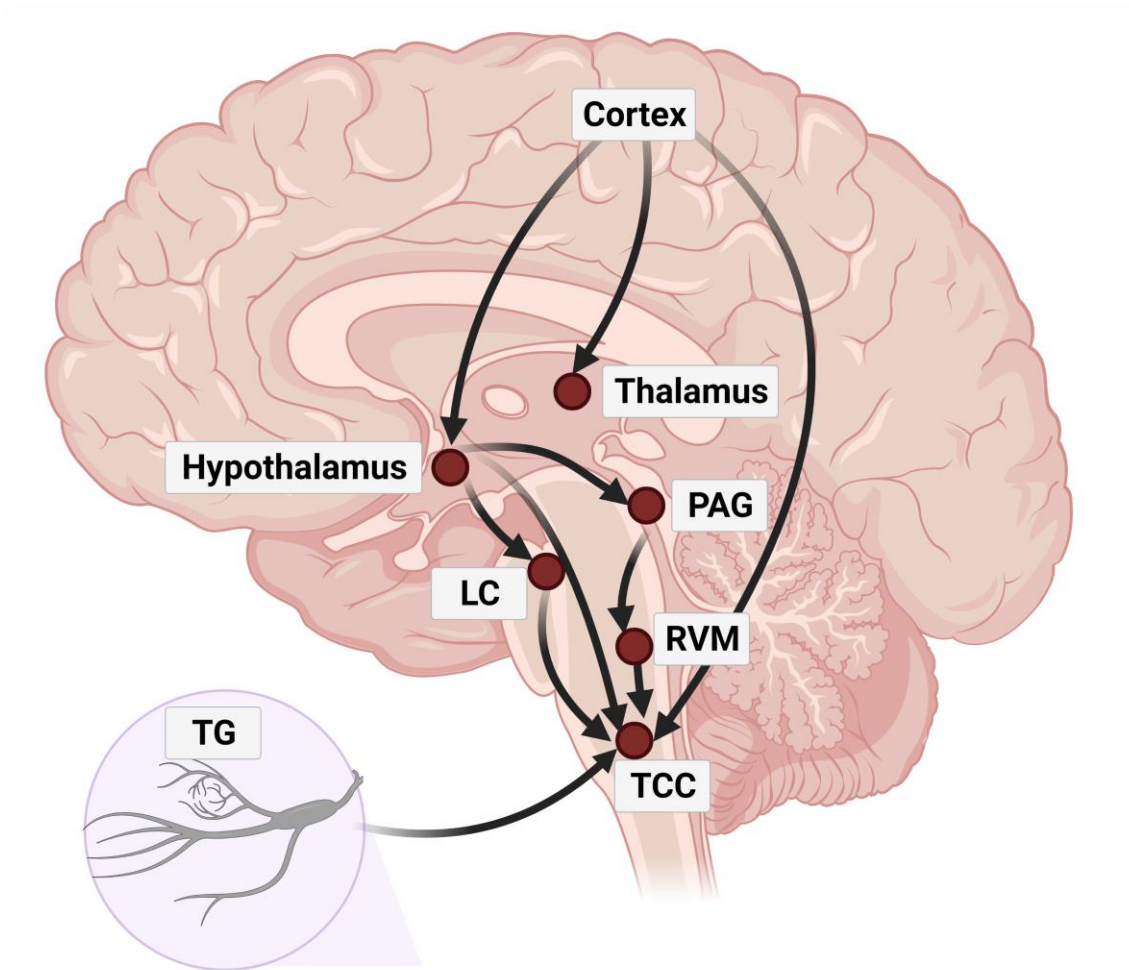
Figure 3: Descending pathways involved in migraine pathophysiology.
Descending trigeminovascular nociception. Adapted from Holland (2014). The trigeminal cervical complex (TCC) receives indirect modulatory descending projections from the brain stem (locus coeruleus; LC; rostral ventromedial medulla; RVM; and periaqueductal grey; PAG), hypothalamus, and trigeminal ganglion (TG). There is also a local corticothalamic circuit which modulates trigeminothalamic processing. The TCC also receives direct projections from the primary somatosensory and insular cortices. This intricate network modulates TCC activity to facilitate or impede nociception.

1.2.4 CGRP

CGRP is a neuropeptide found extensively both centrally and peripherally (Van rossum et al., 1997; Warfvinge & Edvinsson, 2019). In recent years, CGRP been the subject of increased exploration as it was found to play a pivotal role in migraine (Edvinsson & Goadsby, 2019). CGRP is thought to be primarily released by the TG and act upon trigeminal nerve endings and innervate the cranial vasculature, where it acts as a potent vasodilator (Brain et al., 1985; Storer et al., 2004). However, CGRP is not

restricted to the TG as the satellite glial cells also have CGRP receptors, suggesting that CGRP can have a complex array of actions (Edvinsson, 2017, 2019). Of particular importance for migraine is the preponderance of CGRP in trigeminal primary afferents (C-fibres and Aδ-fibres) which synapse in the TCC. CGRP release at these synapses is suggested to increase nociceptive transmission. For example, delivery of CGRP onto nociceptive-responsive TCC neurons in the cat results in significant neuronal excitation (Storer et al., 2004), highlighting a neuromodulatory effect of CGRP in the TCC.

Increased plasma, serum, cerebrospinal fluid (CSF) and saliva CGRP levels have been demonstrated in patients with episodic and chronic migraine (Bellamy et al., 2006; Cernuda-Morollón et al., 2013; Goadsby et al., 1990; Goadsby & Edvinsson, 1993), and CGRP infusion has been demonstrated to trigger migraine-like headache (Hansen et al., 2010; Lassen et al., 2002). Recent therapeutic avenues have focused on CGRP-based treatments (see section 1.2.5), while existing therapies have been shown to reduce CGRP release (Juhasz et al., 2005; Stepień et al., 2003).

1.2.5 Pharmacological Treatment

Pharmacological treatment for migraine is commonly divided into acute and preventive (prophylactic; de Vries et al., 2020). Whilst many treatments have been developed which are specific for migraine (see **Figure 4**), drugs which were originally developed for other conditions are commonly used in the preventive treatment of migraine. These include: antihypertensives (propranolol, candesartan), tricyclic antidepressants (amitriptyline), anti-emetics, anticonvulsants (topiramate), calcium channel blockers (flunarizine), and herbal and nutritional supplements (Urits et al., 2019). However, these are often accompanied by intolerable side effects or contraindications (Goadsby, Lipton, et al., 2002). The following section gives an

overview of the medications used in standard clinical care for acute and preventive treatment of migraine attacks.
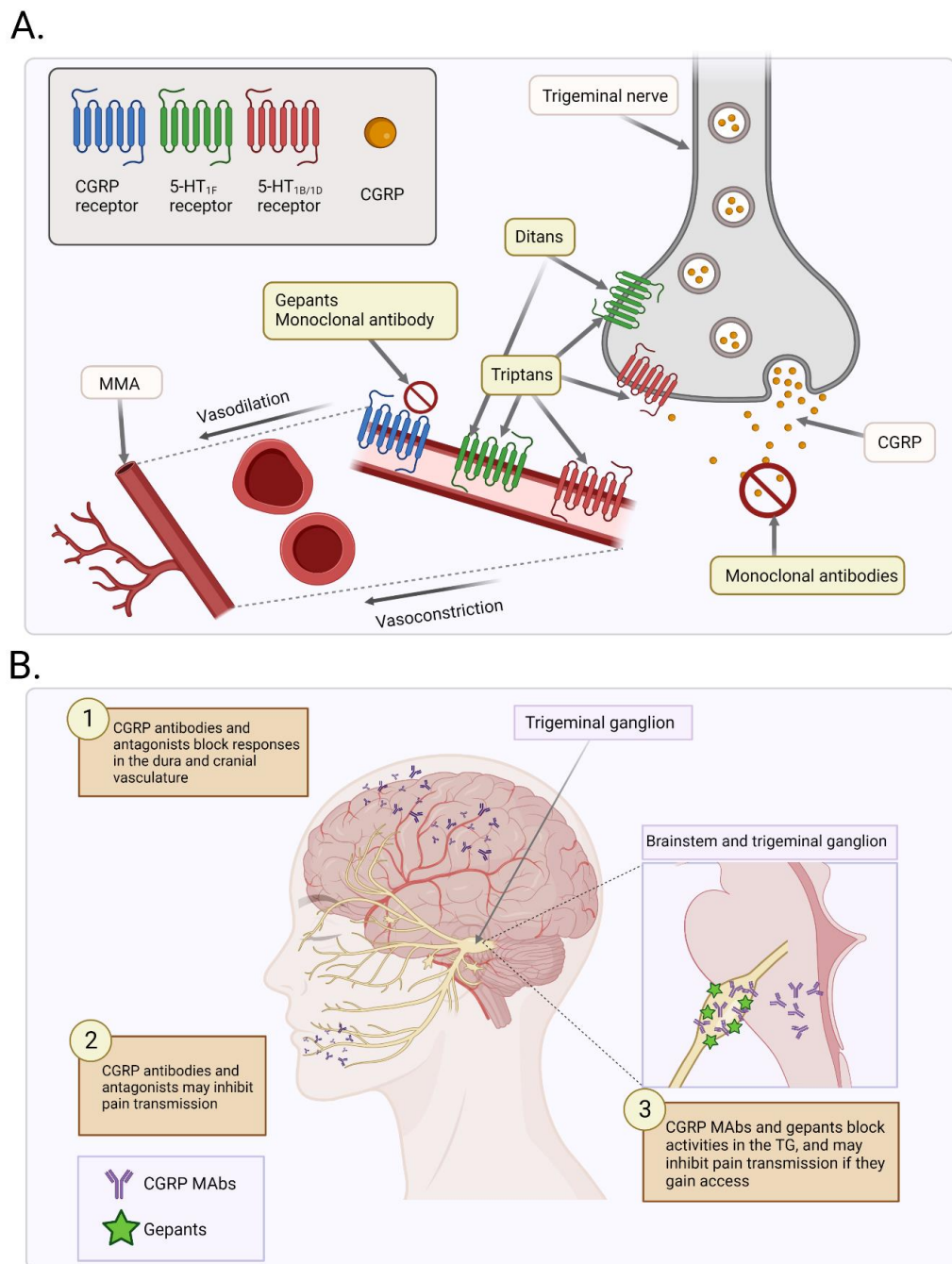
Figure 4: Migraine-specific treatments and potential sites of action
A) Adapted from de Vries et al. (2020). Anti-migraine drugs can work by blocking CGRP release or its receptor, or by activating $5\text{-}HT_{1F}$ or $5\text{-}HT_{1B/1D}$ receptors. MMA = middle meningeal artery; CGRP = calcitonin gene-related peptide. **B**) Adapted from Edvinsson (2017). Schematic of CGRP-based therapies highlighting where the CGRP monoclonal antibodies (MAbs), CGRP receptor antibodies, and CGRP receptor antagonists (gepants) have their main site of action. TG = trigeminal ganglion.

Preventive treatments are used to reduce the frequency, severity, and duration of attacks as well as reduce the need for acute treatments. CGRP monoclonal antibodies which block CGRP signalling by binding directly to the ligand or its receptor have been shown to be effective in preventing migraine (Olesen et al., 2004). Monoclonal antibodies which block the CGRP receptor at present include erenumab (Goadsby, Reuter, et al., 2017; Lattanzi et al., 2019). Whereas fremanezumab (Silberstein et al., 2017), galcanezumab (Lamb, 2018), and eptinezumab (Baker et al., 2017) block the CGRP ligand directly to prevent its binding to the CGRP receptor.

Acute Treatment

Over the counter medications often used in acute treatment of migraine attacks include analgesics (paracetamol) and non-steroidal anti-inflammatories (NSAIDs) (Ashina et al., 2021). However, considered the gold standard for acute migraine treatment are $5\text{-HT}_{1B/1D}$ receptor agonists – known as triptans. Yet these are not effective in 15-40% of patients (Kuca et al., 2018) and have sides effects and cardiovascular contraindications (Negro et al., 2018). Ditans (e.g. lasmiditan) which target the 5-HT_{1F} receptor were later developed which have been shown to be effective and pose less cardiovascular risks (Viticchi et al., 2022). CGRP receptor antagonists known as gepants (for example: ubrogepant (Dodick et al., 2019); atogepant (Ailani et al., 2021); rimegepant (Lipton et al., 2019); zavegepant (Croop et al., 2021)) can be used in the acute treatment of migraine attacks and have been shown to have a similar efficacy to triptans in terms of reducing monthly migraine days, yet are better tolerated (Moreno-Ajona et al., 2020; Negro & Martelletti, 2019, 2021).

Despite the array of pharmacological treatments available for migraine prevention and acute treatment, there remains issues of lack of efficacy and poor

tolerability in many patients (Diener et al., 2015). Indeed, even the most effective preventive medications only reduce headache frequency by 50-60% (Burch, 2021), thus further research into the underlying mechanisms of migraine and identification of novel potential pharmacological targets is warranted.

1.3 Sleep and Migraine

There is a bidirectional clinical association between sleep and headaches which has been recognised since the 19th century (Liveing, 1873; Romberg, 1853). A plethora of evidence has since described the association between alterations in sleep (timing, duration, deviation from typical cycle) and worsening headache symptoms (Dodick et al., 2003; Jennum & Jensen, 2002). For example, sleep has been reported to be a relieving and/or exacerbating factor for headache in 50% of patients (Kelman, 2007), and poor sleep is highly prevalent in migraine and highlighted as key risk factor for chronification (Bigal & Lipton, 2006a; Calhoun et al., 2006). Similarly, headaches, noxious stimuli, and pain disorders affect sleep, and sleep disturbance affects pain perception (Smith & Haythornthwaite, 2004). Equally, headaches can be a symptom of sleep disorders and nocturnal headaches can cause poor sleep (Dodick, 2000). Therefore, headache and poor sleep could be manifestations of a common underlying physiological or systemic dysfunction (Dodick et al., 2003).

Given the aforementioned need for novel therapies, there is a necessity to explore potential underlying mechanisms which may increase migraine susceptibility. As sleep shares a strong association with migraine, it is likely that understanding this relationship further could shed light on migraine pathophysiology. However, to gain understanding of this complex relationship we must first outline the current understanding of sleep regulation and mechanisms.

1.4 Sleep

One third of an adult human's life will be spent asleep, and nearly all animals exhibit some form of sleep (Siegel, 2008). It is argued that sleep must serve an important evolutionary function given lengthy periods of inactivity could be hazardous to survival (Siegel, 2022). Despite this, the function of sleep is relatively poorly understood. Research has demonstrated the importance of sleep for physical and mental wellbeing. More specifically, adequate sleep has been shown to regulate glucose metabolism (Spiegel et al., 2009), cardiovascular health (Malhotra & Loscalzo, 2009), cognition (Harrison & Horne, 2000; Maingret et al., 2016; Peter-Derex, 2019), as well as reduce obesity (Spiegel et al., 2009), diabetes (Lee et al., 2017), mental illness, and psychiatric disorders (Alvaro et al., 2013; Franzen & Buysse, 2017). Sleep also plays a role in the glymphatic system and functions to clear out waste products from the brain (Iliff et al., 2012; Mendelsohn & Larrick, 2013; Xie et al., 2013), with poor sleep being linked to the build-up of β-amyloid plaques in Alzheimer's disease (Di Meco et al., 2014; Lucey & Bateman, 2014). Sleep deprivation has been found to increase the risk of chronic pain and illness, and total sleep deprivation can be fatal (Gallassi et al., 1996; Smith & Haythornthwaite, 2004).

Despite its importance we are thought to be in the midst of a global 'poor sleep epidemic' (Stranges et al., 2012). A survey across 12 countries found that 44% of people reported a decline in sleep quality over the past 5 years and only 40% of those had sought help from a medical professional (Philips, 2019). Similar to migraine, the impact of poor sleep on society has been dubbed a socio-economic crisis with an estimated toll on global health resources of $680 billion each year (Hafner et al., 2017). This is a result of either indirect (workplace safety, road accidents) or direct causes (diabetes, obesity). Indeed, clinicians are beginning to prescribe sleep interventions

and provide sleep hygiene guidance as part of standard care (Calhoun & Ford, 2007; Moloney, 2017; Torrens et al., 2016).

1.5 Physiology of Sleep

1.5.1 Initiation of Sleep

Like the pathophysiology of migraine, the physiology of sleep is complex. The initiation, timing, depth and duration of sleep is thought to involve two separate mechanisms: circadian and homeostatic (Borbély, 1982; Borbély et al., 2016; see **Figure 5**). The circadian component ('process C') functions independent of activity and is regulated by a pacemaker in the suprachiasmatic nucleus (SCN) situated in the hypothalamus. It aids in synchronising sleep-wake patterns to appropriate times of the day. At the molecular level, neurons in the SCN contain a genetically driven clock, with a transcriptional-translational feedback loop that ensures an approximately 24 hour cycle (Saper, Lu, et al., 2005), which involves positive regulatory genes: Circadian Locomotor Output Cycles Kaput (CLOCK), Brain and Muscle ARNT-like 1 (BMAL1); and negative regulators cryptochrome (CRY1-2) and period (PER1-3) (Landgraf et al., 2012; see **Figure 6**).

The SCN releases diffusible agents which are capable of sustaining the circadian rhythm but require input from SCN tissues to sustain this in relation to light (Conduit & Robinson, 2017; Iglesia et al., 2003; Lupi et al., 2008; Silver et al., 1996). The conversion between the circadian rhythm and external environmental factors such as light is termed entrainment. To facilitate entrainment, the SCN receives direct and indirect projections from the retina (Abrahamson & Moore, 2001). The direct projections include those with intrinsically photosensitive (melanopsin) retinal ganglion cells, whereas the indirect projections influence the SCN via the thalamus (Hattar et al., 2002; Zele et al., 2011). During darkness, the SCN receives melatonin

from the pineal gland to entrain the clock (Dubocovich, 2007). If the circadian rhythm is allowed to run in the absence of light, it averages 24 hours, 9 minutes ± 12 (Duffy et al., 2011).
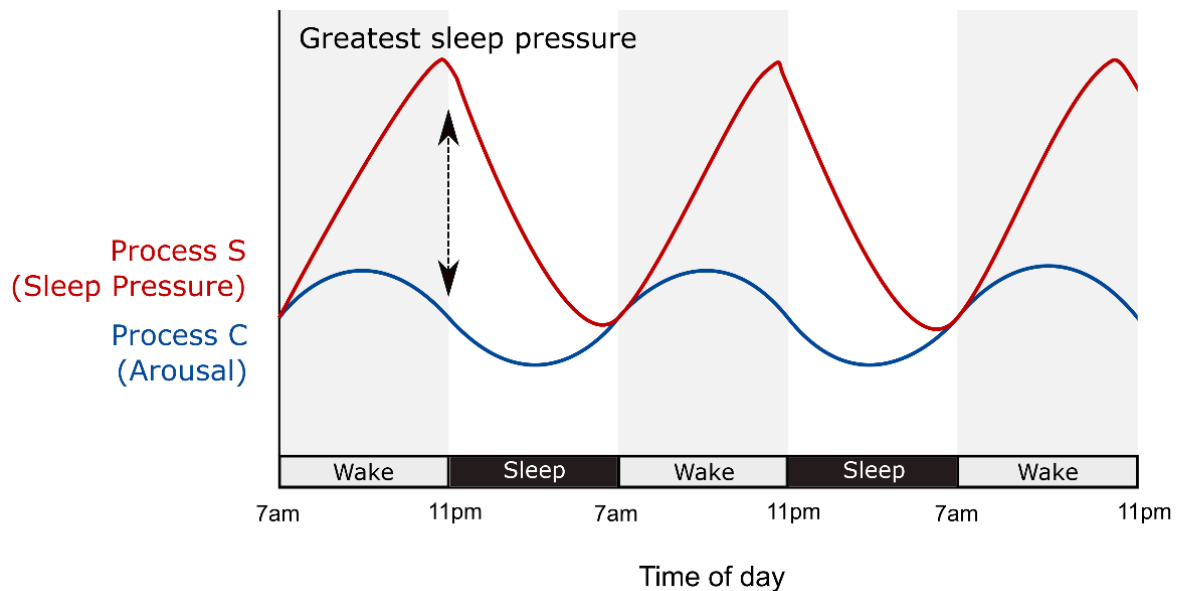
Figure 5: Borbély's two-process model of sleep regulation
A representation of process "S" – homeostatic sleep pressure, and process "C" – circadian arousal according to Borbély's (1982) two process model of sleep regulation. The point at which the discrepancy between high sleep pressure and low circadian arousal is greatest is indicated by the dashed black arrow.

The homeostatic component ('process S') refers to the need to sleep due to increasing time awake i.e. the longer the duration of prior wakefulness, the greater the need to sleep (although other factors can alter this need, see Fredholm (2014); Ganguly-Fitzgerald et al. (2006)). The neurochemical correlate of this is adenosine - an endogenous somnogen generated from adenosine triphosphate (ATP) which accumulates throughout the day predominantly in the basal forebrain and declines during recovery sleep (Porkka-Heiskanen et al., 1997). Indeed, injections of adenosine result in promotion of sleep (Dunwiddie & Worth, 1982). In support of this two-process model, lesions of the SCN results in loss of circadian activity patterns of rest and activity yet sleep pressure or the homeostatic drive to sleep remains intact (Achermann, 2004).
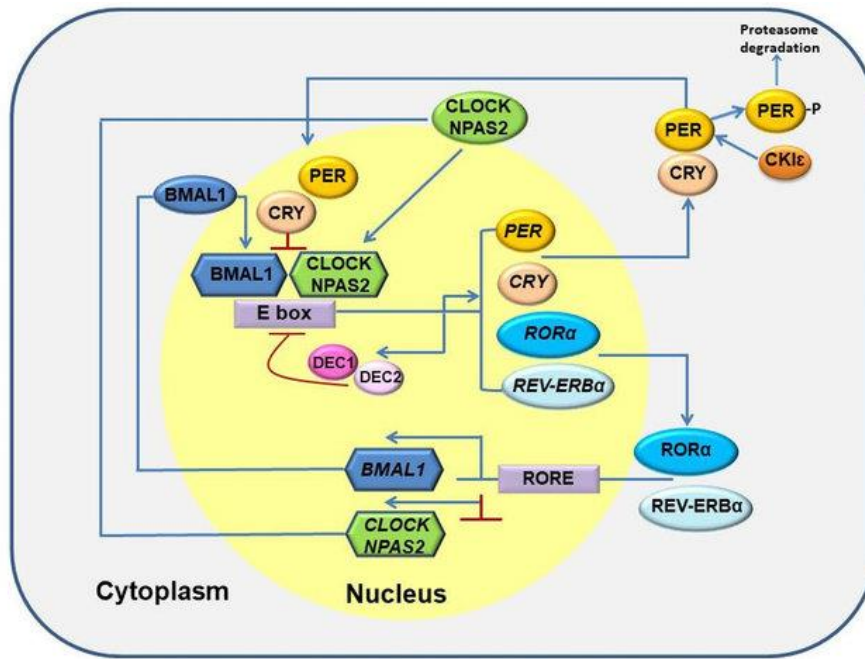
Figure 6: Genes involved in the regulation of circadian rhythm and sleep
Taken from Rahman et al. (2019). The core circadian clock involves a TTFL loop comprising CLOCK and BMAL1. DEC2 and DEC1 are also involved in the regulation of these clock genes by acting on these E-box elements.

When sleep need reaches a certain threshold and timing is consistent with circadian phase (process C), sleep is initiated by high levels of adenosine which activates the sleep-promoting neurons in the ventral lateral preoptic nucleus (VLPO) of the anterior hypothalamus which contain Gamma-Aminobutyric acid (GABA) and galanin (an inhibitory peptide) (see **Figure 7**). Neurons in the VLPO along with those in the brainstem inhibit the arousal-associated cholinergic neurons in the basal forebrain (Sherin et al., 1996). The VLPO consists of two main groups of neurons, one projecting to the tuberomammillary nucleus (TMN), and another to the LC and dorsal median raphe nuclei (DMR) (Fuller et al., 2006). These regions form part of the ascending reticular activating system (ARAS) (Mesulam, 1995) and additionally include the pedunculopontine tegmentum (PPT), and lateral dorsal tegmentum (LDT) (Brennan & Charles, 2009). This suppression leads to a reduction in ascending afferent activity reaching the cortex and sleep is initiated.

If sufficient sleep is not obtained, then a sleep rebound will often occur in which there is a compensatory increase in the amount and depth of sleep, typically characterised by increased power spectral density (PSD) in the delta range (1-4Hz) during subsequent sleep (Agnew et al., 1964). Thus, delta power during sleep is commonly used as a quantitative measurement of process S.
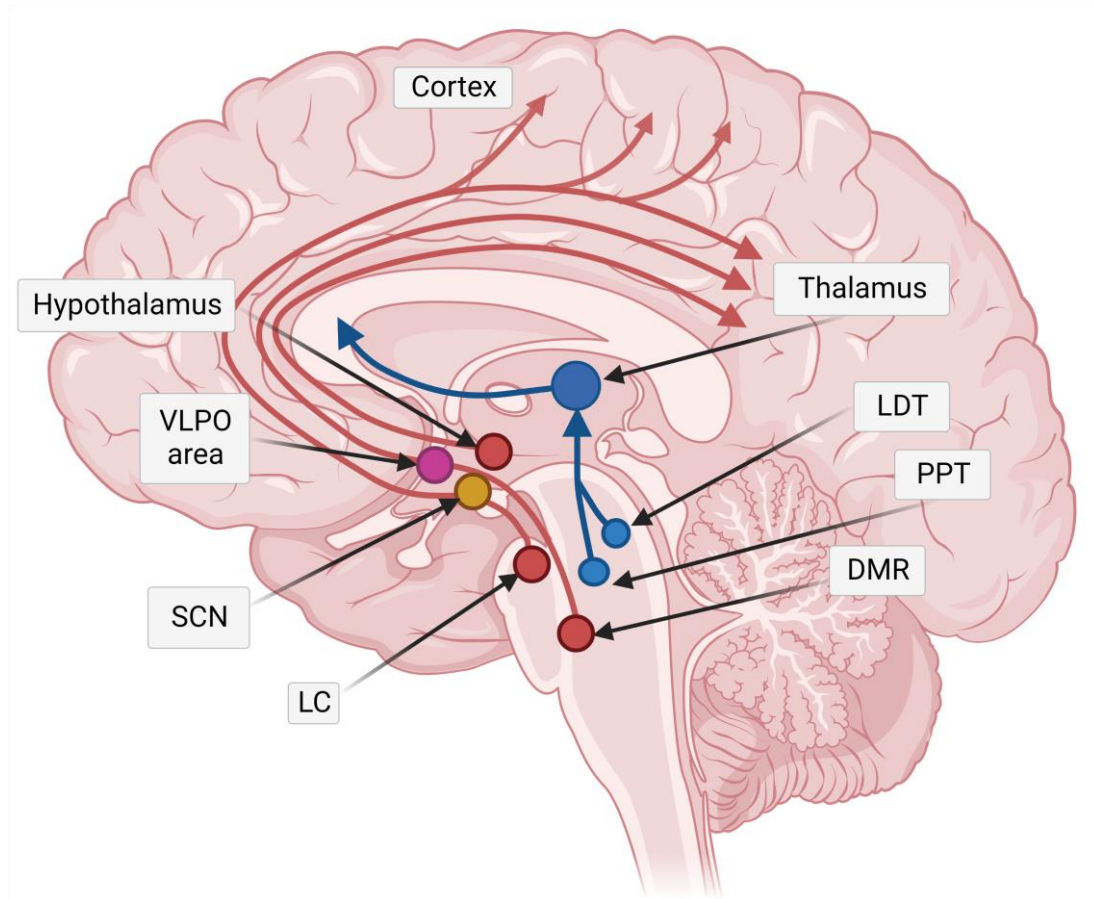
Figure 7: Key brain regions involved in sleep regulation
The ascending reticular activating system (ARAS) is made up of a complex network of regions which regulate sleep and arousal. The SCN (yellow) of the hypothalamus plays an important role in the generation of circadian rhythm and synchronisation of the biological clock. The VLPO area (purple) and brain stem regions (LC, DMR) inhibit the arousal-associated cholinergic neurons in the basal forebrain. This suppression leads to a reduction in ascending afferent activity reaching the cortex. Regions in blue indicate the thalamic pathway of the ARAS. The LDT and PPT cholinergic neurons project to the thalamus to control thalamic sensory gating. Regions in red indicate the hypothalamic monoaminergic pathway which innervates the cortex to produce arousal. Abbreviations: SCN = suprachiasmatic nucleus; VLPO area = ventrolateral preoptic nucleus of the hypothalamus; LDT = lateral dorsal tegmentum; PPT = pedunculopontine tegmentum; LC = locus coeruleus; DMR = dorsal median raphe nuclei.

1.5.2 Cessation of Sleep

When the markers which signal sleep need (e.g. adenosine, somnogenic cytokines) are at their lowest level, the neural systems inhibited by the VLPO to promote sleep are involved in promoting wakefulness (Saper & Fuller, 2017). The ARAS begins in the upper pons and contains two branches – one cholinergic pathway initiated in the LDT and PPT which innervates the thalamus via inhibitory GABA connections. This in turn activates thalamic relay neurons which project to the cerebral cortex. The other, monoaminergic pathway projects to the lateral hypothalamus (LH), basal forebrain and cerebral cortex and includes the noradrenergic LC, serotonergic DMR, dopaminergic PAG and histaminergic TMN (Fuller et al., 2011). There are also two important pathways from the LH which ensure arousal: orexinergic (also known as hypocretin) neurons which stimulate the LC, TMN, and DRM, and melanin concentrating hormone (MCH) which projects to similar structures (Fuller et al., 2006).

1.5.3 Sleep-wake Transition

Saper et al. (2005) proposed the 'flip-flop' switch for explaining how smooth and abrupt transitions occur between sleep and wake, disfavouring transitional states (see **Figure 8**). The switch which is thought to reside in the hypothalamus can abruptly promote sleep and inhibit wakefulness and vice versa. As mentioned previously, during wake, the hypothalamus innervates two pathways – one which consists of cholinergic brainstem nuclei that activate the cortex via the thalamus, and one composed of brainstem structures which broadly project to the cortex. To ensure transitions between sleep and wake are not too abrupt and frequent, the excitatory orexinergic neurons which reside in the lateral and posterior hypothalamic nuclei increase the firing rate in the TMN, LC and DMR, and indirectly inhibit the VLPO

(Gompf & Anaclet, 2020) to reinforce and stabilise the waking state and promote cortical desynchrony (Alexandre et al., 2013). If this switch does not function correctly, it can cause changes to the sleep cycle. For example, orexin knock-out mice display reduced consolidation of both sleep and wake (Chemelli et al., 1999) – the main dysfunction in patients with the sleep disorder narcolepsy. Additionally, if one side of the switch is weakened, such as with VLPO cell loss in the elderly, this can result in sleep fragmentation and more frequent diurnal naps (Saper, Scammell, et al., 2005).
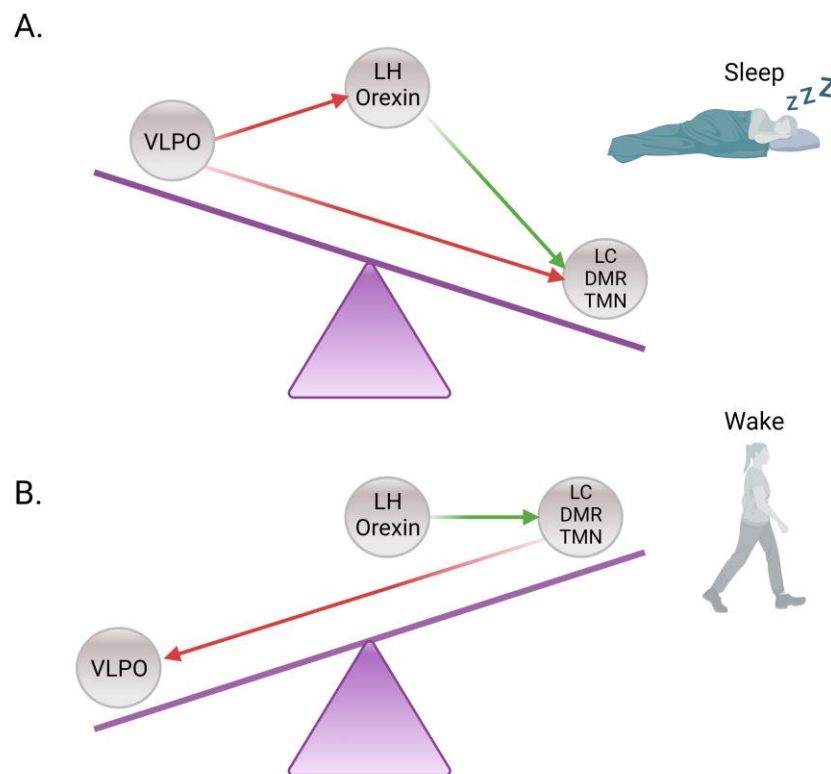
Figure 8: The flip-flop switch of sleep-wake transition
Red arrows indicate inhibitory connections and green arrows indicate excitatory connections. A = during sleep the VLPO area inhibits the monoaminergic cell groups including the LC, DMR, and TMN, and the orexinergic LH. B = during wake, the LC, DM raphe nucleus and TMN inhibit the VLPO area and orexin stabilises the waking state. Abbreviations: VLPO, ventral lateral preoptic nucleus; LH, lateral hypothalamus; LC, locus coeruleus; DMR, dorsal median raphe nucleus; TMN, tuberomammillary nucleus.

1.5.4 Sleep Cycles and Stages

Under modern societal pressures, humans typically have quasi-monophasic sleep patterns - sleeping for a single nocturnal period with the occasional diurnal nap (Stampi, 1992). However, 86% of mammals exhibit biphasic or polyphasic sleep where sleep occurs in two or more consolidated periods of time (Campbell & Tobler, 1984). Although, evidence exists that human populations isolated from modern civilisation exhibit highly polyphasic sleep (Petre-Quadens, 1983). In humans, sleep usually changes from polyphasic during infancy to become more monophasic in early childhood (Chokroverty, 1994; Roffwarg et al., 1966).

In mammals, sleep is typically characterised into non-rapid-eye-movement sleep (NREM) and rapid-eye-movement (REM) sleep (also known as paradoxical sleep). In humans, sleep stages follow an ultradian pattern and cycle every 90 minutes in which NREM sleep is followed by REM sleep (see **Figure 9**; Saper et al., 2010). The length of individual sleep cycles, as well as the cycle itself, varies considerably across mammals with a tendency for shorter NREM-REM cycles in smaller animals (Chokroverty, 1994). For example, a typical sleep cycle is 12 minutes in the laboratory rat (McCarley, 2007) . The percentage of each sleep stage within the sleep period, and the length of each sleep stage episode also varies across mammalian species (Van Twyver, 1969). The proportion of sleep stages also changes over the nocturnal period; the deeper stages of NREM – slow-wave sleep (SWS) predominate early in the night, whereas REM sleep dominates in the latter half of the night (Roth & Roehrs, 2000). Sleep macro-architecture changes with age, with a tendency for less SWS, more frequent arousals and diurnal naps in the elderly (Dijk et al., 2010). REM sleep takes up 50% of all sleep time in new-born infants and this percentage declines throughout childhood to be around 20% during adulthood (Jenni & Carskadon, 2005). Sleep cycles

can also change dependant on other factors including prior sleep deprivation, various psychiatric and physiological conditions, and medications (Orzeł-Gryglewska, 2010).
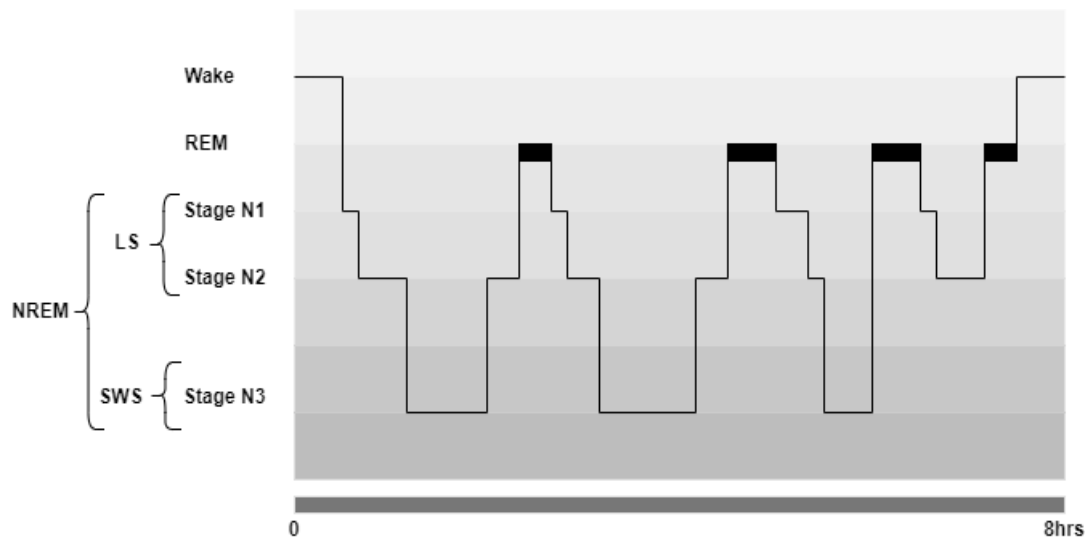
Figure 9: Hypnogram of sleep cycles in a healthy adult human
Sleep cycles follow a regular ultradian pattern lasting around 90 minutes each, beginning with light stages of NREM sleep before progressing to deeper NREM (SWS) and eventually into REM sleep. Abbreviations: REM = rapid-eye-movement sleep; LS = light sleep; NREM = non-rapid-eye-movement sleep; SWS = slow-wave sleep. Adapted from: Genzel et al. (2014).

NREM sleep has increasing levels of depth as characterised by the appearance of the cortical electroencephalogram (EEG), with stage 1 being lighter, moving toward stage 3 which is sometimes known as deep sleep or SWS (see **Figure 10**). Humans typically spend 5% of the night in stage 1 NREM sleep, 20% in SWS, 50% in stage 2 NREM, and 20-25% in REM (Erwin et al., 1984). SWS is characterised by high amplitude, low frequency oscillations in the delta range (1-4 Hz) and relatively little response to environmental stimuli as sensory input to the cortex is limited via thalamic gating (Chauvette, 2013; Rechtschaffen et al., 1966). Such slow oscillations (SO) during SWS are thought to be generated by cycling of up and down-states which reflect synchronised neuronal depolarisation and hyperpolarisation respectively (Timofeev et al., 2001) and are largely synchronous across all cortical regions. However, SOs can

also occur more locally such as in response to a particular event or process (Huber et al., 2006).

In stage 2 NREM sleep, slow (12-14 Hz) and fast sleep spindles (14-16 Hz oscillations, ~1 second) dominate, whose function is thought to be inhibiting sensory information from reaching the cortex, preventing arousal (De Gennaro & Ferrara, 2003; see **Figure 11**). Although recent evidence challenges this view and suggests they may be involved in facilitation of information including cortical nociception (Claude, Chouchou, Prados, Castro, De Blay, et al., 2015). Like SOs, spindles show global coherence across thalamic and neocortical regions (Contreras et al., 1996), but they are thought to be generated by GABAergic interneurons in the thalamic reticular nucleus (Steriade, 1995). Alterations in sleep spindle frequency and distribution have been associated with psychotic, neurodegenerative, and neurological disorders such as epilepsy and schizophrenia (Clawson et al., 2016; Wamsley et al., 2012).

Stage 2 NREM sleep is also hallmarked by K-complexes (see **Figure 11**). K-complexes are events which typically occur in SWS at the frequency of SOs. They have two main components: a positive wave which reflects synchronous excitation of neurons, and a negative wave reflecting neuronal hyperpolarisation (Wauquier et al., 1995). They can occur spontaneously or in response to sensory stimuli. K-complexes are thought to be involved in triggering and co-ordinating other neuronal oscillations (Amzica & Steriade, 2002) including sleep spindles and delta waves.

REM sleep on the other hand is characterised by low amplitude mixed frequency oscillations in the theta range (4-7 Hz), skeletal muscle atonia, phasic muscle twitches, dreaming, and rapid movements of the eyes (Berry, 2015). The first REM period of the night tends to occur 45-90 minutes after the onset of sleep in humans, with the first REM period being short (~5-10 minutes). REM sleep is linked with long-term memory

ability (Siegel, 2001), growth (Schmidt, 2014), chromosome repair (Zada et al., 2019) and alterations in the proportion of REM sleep and REM sleep latency have been linked to mental illness, psychiatric, and neurological disorders (Pandi-Perumal & Kramer, 2010).
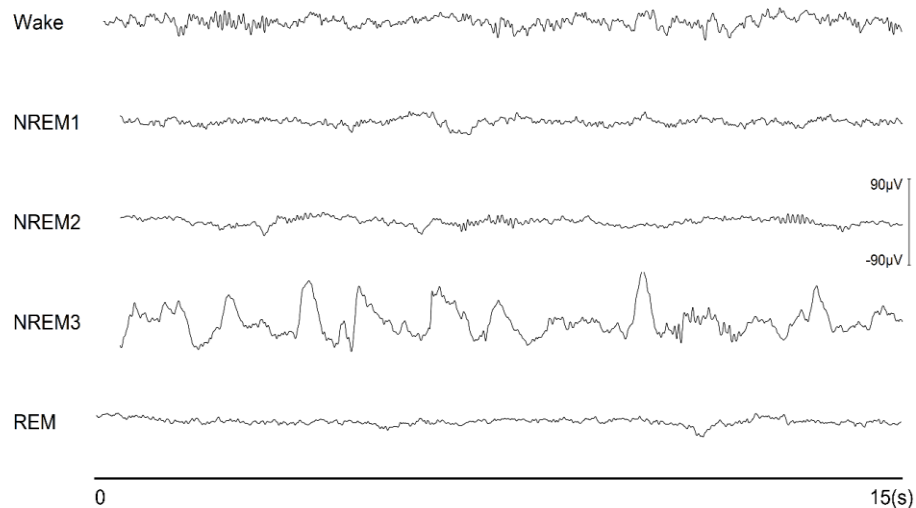
Figure 10: Neural oscillations during sleep stages
A healthy adult individual's sleep captured on a single EEG channel (Fpz). Abbreviations: NREM1, Non-rapid-eye-movement sleep stage 1; NREM 2, Non-rapid-eye-movement sleep stage 2; NREM3, Non-rapid-eye-movement sleep stage 3; REM, rapid-eye-movement sleep.
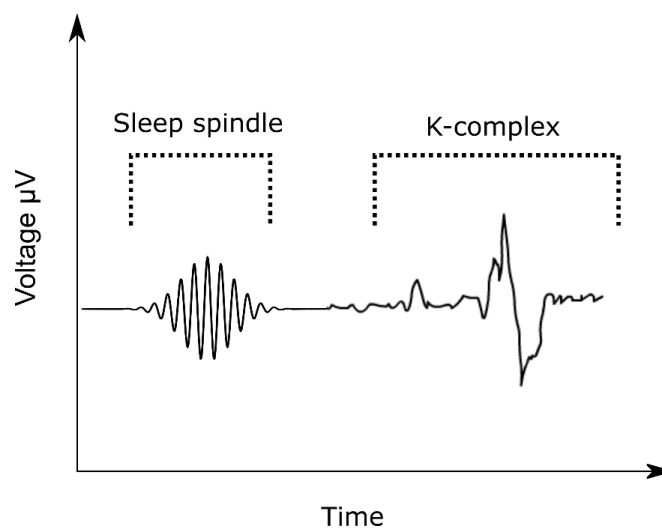
Figure 11: Neural oscillations during NREM sleep
Schematic of a sleep spindle and K-complex as seen on a typical polysomnogram in adult humans during stage 2 non-rapid-eye-movement sleep.

1.5.5 Sleep Stage Transition

The transition between sleep stages is abrupt and thought to be regulated by its own flip-flop switch (see **Figure 12**). For example, Lu et al. (2006) propose that REM sleep is controlled by a REM-ON/REM-OFF switch composed of mutually inhibitory GABAergic projections between REM sleep inhibiting (REM-OFF) neurons in the vlPAG (Sapin et al., 2009), lateral pontine tegmentum (LPT), LH and DMR, and REM sleep promoting (REM-ON) cholinergic neurons in the sublaterodorsal tegmental nucleus (SLD), lateral oral pontine reticular nucleus, and the pedunculopontine tegmental (PPT) nuclei (Luppi et al., 2012; Sastre et al., 1996). Whereas the dorsomedial hypothalamus is thought to play a role in NREM/REM transitions via galaninergic neurons (Chen et al., 2018).
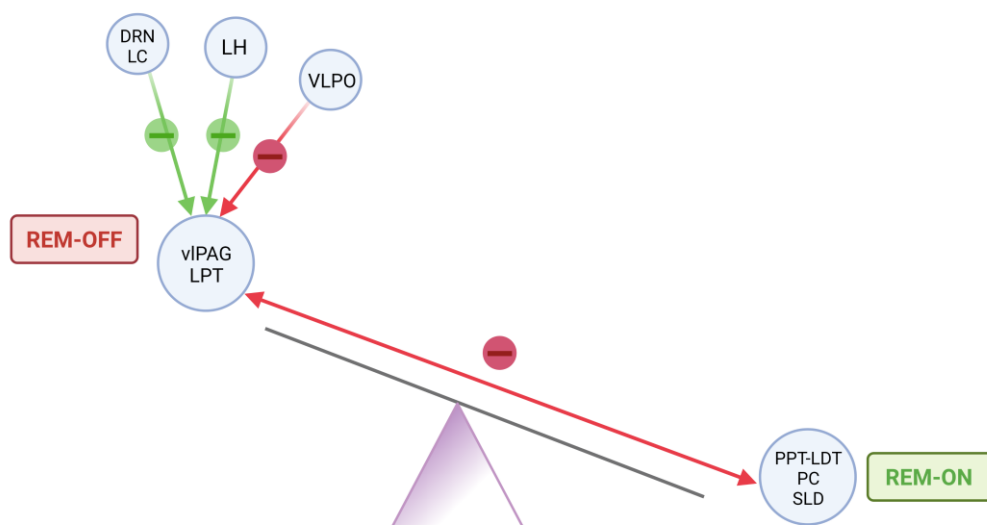
Figure 12: The flip-flop switch for NREM-REM sleep stage transitions
Lu et al. (2006) proposed a flip-flip switch for sleep stage transitions. REM-OFF neurons in the vlPAG and LPT exhibit direct mutually inhibitory connections with REM-ON GABAergic neurons in the SLD and PC. Orexinergic neurons in the LH also show indirect inhibitory connections with the REM-ON regions. and DRN and LC neurons activate REM-OFF circuitry but are not directly inhibited by REM-ON regions. REM = rapid-eye-movement sleep; vlPAG = ventrolateral periaqueductal gray; LPT = lateral pontine tegmentum; PC = precoeruleus, SLD = sublaterodorsal nucleus; LH = lateral hypothalamus; DRM = dorsal raphe nuclei.

1.5.6 Variations in Circadian and Sleep timing

Despite the average circadian rhythm duration in humans being approximately 24 hours, 9 minutes ± 12 (Duffy et al., 2011; see section 1.5), the duration of an individual's circadian oscillation (tau; τ) displays significant variation across the population (Ashbrook et al., 2020). How an individual's rhythm aligns with day or night also exhibits variation and this is termed chronotype (Roenneberg, 2012). Chronotype follows a normal distribution and can be affected by many factors for example gender, age (Hu et al., 2016), and geographical location (Miguel et al., 2014). Individual variation in chronotype is thought to be largely driven by genetic factors (Kalmbach et al., 2017), and these variations have been shown to impact on behavioural and health outcomes (Jones et al., 2019; Suh et al., 2017). For example, migraine patients have been shown to exhibit chronotypes at the opposite ends of the normal distribution with most having an early chronotype (~50%) or late (40%; (Gori et al., 2005; van Oosterhout et al., 2018). Furthermore, exhibiting a morning chronotype is predictive of migraine chronification and attack severity and frequency (Sullivan & Martin, 2017; Viticchi et al., 2019), highlighting the importance of investigating circadian rhythmicity in health and disease.

Although most healthy individuals present with an intermediate chronotype, extreme phenotypes can occur which can result in sleep/wake disorders (see **Figure 13**). For example, advanced sleep phase (ASP) or 'morning larks' are individuals which prefer to sleep, and wake early (Fu & Ptáček, 2019). Conversely, individuals with delayed sleep phase (DSP) or "night owls" prefer to go to sleep late and awaken late. Although, these variations are not usually cause for concern, if they become pathological this is known as advanced sleep–wake phase disorder (ASWPD) and delayed sleep–wake phase disorder (DSWPD) respectively (Darien, 2014).

As well as variations in circadian alignment, homeostatic sleep need varies within the population (Klerman & Dijk, 2005), and has a prominent genetic basis (Franken et al., 2001). This can be split into habitual long or short sleepers (Aeschbach et al., 2003; Allebrandt et al., 2010). Recent studies have identified some individuals as familial natural short sleepers (FNSS) who sleep around 4-6 hours a night; and there are a number of reported genetic mutations implicated in FNSS (He et al., 2009; Shi et al., 2019, 2021; Xing et al., 2019). Importantly, FNSS are set apart from facultative short sleepers who may require more sleep but exhibit shorter sleep duration due to social constraints, and experience typical sleep-deprivation-induced cognitive difficulties or drowsiness (Yook et al., 2021).

Recent evidence suggests reduced cognitive difficulties following sleep deprivation (Xing et al., 2019), altered immune function (Fondell et al., 2011) and reduced Alzheimer's-related pathology (Dong et al., 2022) in human carriers and mice carrying mutations for FNSS. Moreover, human FNSS are thought to have relative resistance to pain and life stressors (personal communication, Professor Louis Ptáček, 2022), suggesting that natural variations in sleep need may be protective.
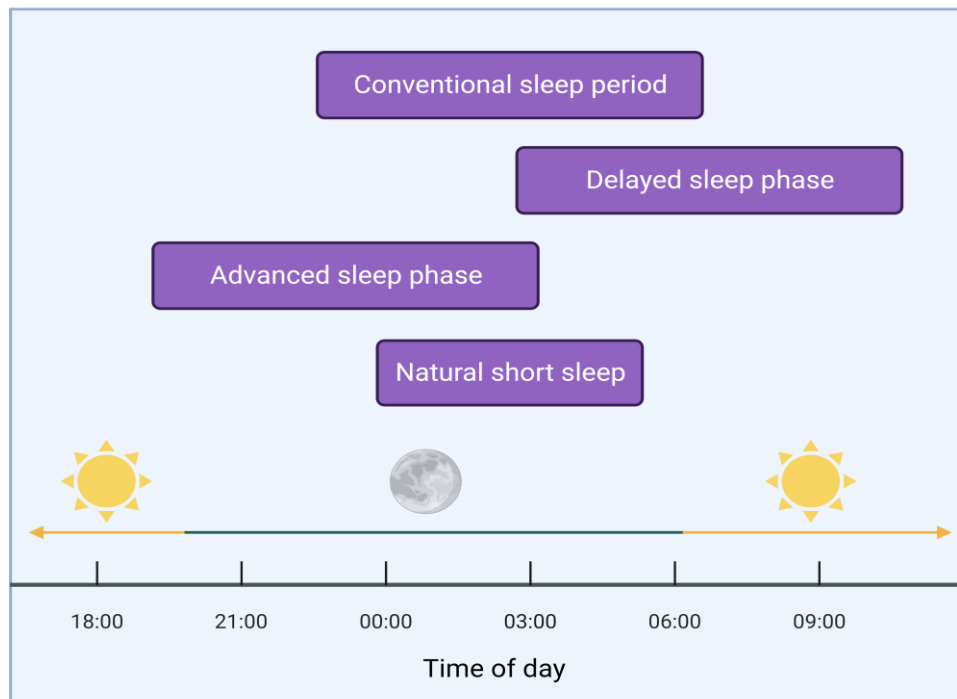
Figure 13: Variations in sleep timing.
The conventional human sleep period (~8 hours) compared with delayed (late sleep onset) and advanced sleep phase (early sleep onset) and natural short sleepers (4-6 hours, early offset). Adapted from Ashbrook et al. (2020).

1.6 Sleep and Migraine: Clinical Relationship

As touched upon previously, there is a complex clinical relationship between sleep and migraine. This picture is further complicated by the fact that several treatments for migraine affect the sleep cycle (e.g. tricyclic antidepressants, serotonin antagonists; Nesbitt et al., 2014), and co-morbid affective disorders involving sleep disturbance are three times more likely in people with migraine than the general population (Hamelsky & Lipton, 2006), and manipulations to sleep can have profound effects on other neural systems (Cirelli et al., 2004), making it difficult to establish a direct relationship.

To explore this relationship in more detail, sleep deprivation or disturbance is a commonly reported trigger for migraine (Andress-Rothrock et al., 2010; Pellegrino et al., 2018). For example, one study showed that two consecutive days of poor sleep were

strongly predictive of headache, whereas adequate sleep was protective (Houle et al., 2012). Conversely, going to sleep can promote headache and sleeping in excess can also be a trigger (Dexter & Weitzman, 1970; Inamorato et al., 1993). This is not restricted to nocturnal sleep as headaches can follow diurnal naps (Dexter, 1979). A recent study observed that greater headache severity was associated with longer naps in chronic migraine patients, and longer nap duration was associated with lower sleep efficiency (Ong et al., 2023). On the other hand, sleep can provide relief during migraine attacks and many patients report using naps as a coping strategy (Kelman & Rains, 2005; Minatti-Hannuch et al., 1991; Ong et al., 2023; Ong & Park, 2012; Parrino et al., 1986). For example, in one early study, 14 out of 50 migraineurs could 'shorten' their attacks by sleeping 2.5 hours during the day (Blau, 1982).

What's more, there is a clear circadian, circannual, and seasonal component to migraine (Fox & Davis, 1998). For example, most migraine patients report attacks in the early hours of the morning (Park et al., 2018; Solomon, 1992) and often at the same time each day (Baksa et al., 2019), with a greater frequency of attacks reported in the summer months (Alstadhaug et al., 2005). This links with recent findings on the seasonality of sleep architecture whereby the authors demonstrated an increase in REM sleep and shorter REM sleep latency during the winter months in Europe (Seidler et al., 2023), suggesting that migraine may be triggered by alterations in sleep and circadian alignment due to changes in natural light levels. Indeed, migraine patients commonly report circadian misalignment e.g., jet lag and shift-work as key triggers for attacks (Leso et al., 2020; Wöber et al., 2007). Moreover, chronic migraine patients are reported to exhibit circadian misalignment (i.e., a greater phase angle) compared to healthy controls and this is correlated with attack severity suggesting circadian misalignment could be an underlying or exacerbating factor for migraine (Ong et al.,

2018). Predicated on this, previous studies from our laboratory have uncovered that mutations in circadian clock genes (CK1δ and PER2) may be responsible for mediating migraine pathophysiology and susceptibility via altered glutamatergic transmission (Strother, 2020). Notably, this work revealed that that realignment of the circadian clock via pharmacological and behavioural intervention could ameliorate these effects.

Additional observations support the link between sleep and migraine. For example, fatigue is cited as a common premonitory symptom in the 48 hours before a migraine attack and fatigue also occurs in the postdrome (Giffin et al., 2003; Stanic & Sretenovic, 2013). However, it is possible that the natural improvement of migraine is associated with fatigue, which could be seen as a marker of migraine recovery rather than a co-morbid symptom. In addition, sensitivity to light as well as exacerbation of headache pain by light (photic allodynia) are common symptoms experienced during migraine attacks and light strongly regulates our circadian clock, highlighting a potential overlap between sleep, circadian rhythm, and migraine.

Moreover, one study found that women are twice as likely as men to suffer from poor sleep (Madrid-Valero et al., 2017), and migraine is more common in women highlighting a possible association. Interestingly, 20-25% of females with migraine present with menstrual migraine, whereby attacks are associated with menstruation (Maasumi et al., 2017; Vetvik & MacGregor, 2021). Sleep changes during the menstrual phase – with less REM sleep and an increase in sleep spindle activity during stage 2 NREM sleep, whereas SWS remains relatively unchanged (Driver et al., 1996). Many patients report excess daytime sleepiness, although whether this is due to poor sleep as a result of the attack is unclear (Barbanti et al., 2013; Kim et al., 2016; Peres et al., 2005).

Further indication of a link between migraine and sleep is that early morning migraine attacks increase with age and chronic migraine is more prevalent in older age (Bigal et al., 2006; Lipton, 2009). In old age, SWS duration and quality is reduced (Spiegel et al., 2009; Yoon et al., 2003), sleep tends to become more fragmented, and sleep spindles become less frequent and shorter (Clawson et al., 2016), and sleep fragmentation has been linked to increasing odds of headache (Boselli et al., 1998). This may reflect a shared age-related deterioration in brainstem and hypothalamic sleep-promoting mechanisms (Vgontzas & Pavlović, 2018), or age-related changes in circadian rhythms (Chen et al., 2016).

1.6.1 Sleep Macrostructure in Migraine

Headache thus appears to have a strong relationship with sleep. What is unclear from the previous studies however is whether it is a reduction in sleep duration that triggers migraine or simply disruption to the normal sleep cycle. For example, one study examined sleep and migraine onset in migraineurs using polysomnographic (PSG) recordings within the laboratory. Interestingly, they found no relationship between short sleep duration and odds of headache occurrence. However, sleep fragmentation, where patients awoke frequently, was associated with higher odds of migraine the following day (Bertisch et al., 2019). Similarly, a prospective study found that frequent night-time awakenings but not decreased total sleep time (TST) precedes a migraine attack (Vgontzas & Pavlović, 2018). This is supported by the finding that sleep fragmentation but not decreased TST lowers pain thresholds (Smith et al., 2007). Furthermore, migraineurs with sleep-related attacks have been found to have more arousals than those without sleep-related attacks, and they exhibited reduced pain thresholds (Boselli et al., 1998; Engstrøm, Hagen, Bjørk, Gravdahl, et al., 2013).

This raises the question of whether migraines are in part precipitated by disruption in the cyclic pattern or alterations in the proportion or timing of a particular sleep stage. Indeed, migraine has been temporally linked specifically to REM sleep. Many patients report an increased frequency of migraine attacks in the morning when there is an increased proportion of REM sleep (Rasmussen, 1993), and nocturnal migraines tend to occur after being awoken from a REM sleep period (Dexter, 1979; Heather-Greener et al., 1996; Hsu et al., 1977; Sahota & Dexter, 1990). In one study, when circadian phase was artificially advanced by 7 hours, thus disentangling any circadian effects on migraine occurrence, patients reported migraines only when aroused from REM sleep (Dexter & Riley, 1975). Interestingly, aura features in some migraine patients can 'protrude' into the content of dreams during REM sleep and elicit arousal (Dexter, 1979). However, in these studies researchers tend to study the subset of migraineurs whose migraines typically occur once awoken from sleep. However, one study found that only 17% of patients in their clinic had nocturnal or early morning migraine attacks (Paiva et al., 1997), suggesting that in the majority of patients attacks may not be related to REM sleep.

Another issue with these studies is that just because a headache occurs after awakening from a REM period does not mean dysfunction or a cause specific to REM sleep. Whilst this is contested, REM sleep may have a lower arousal threshold than the deeper stages of NREM, therefore participants are more likely to wake and stay awake for longer periods of time during this stage (Ermis et al., 2010; Langford et al., 1974). Migraine could be initiated in another sleep stage but it is not apparent until the individual is awoken from REM sleep (Cirignotta et al., 1983).

REM sleep itself can be affected in migraine patients. One study found that REM sleep quantity and REM latency were reduced in migraineurs (Drake et al., 1990).

Importantly, this was on non-migraine days, suggesting that this is not due to being frequently aroused with migraine or pain. More generally, REM sleep loss has been shown to produce hyperalgesia the next day (Roehrs et al., 2006), and REM sleep deprivation leads to increased mechanical withdrawal thresholds and increased activation in nociceptive transmission areas of the brain (Kim et al., 2019). Although, it is often unclear whether the patients in these studies are currently taking migraine prophylactics drugs, although many drugs prescribed for migraine typically depress REM sleep and reduce REM sleep latency but do not affect other stages (Nesbitt et al., 2014; Wilson & Argyropoulos, 2005). Hence, the medication used to treat migraine could be affecting REM sleep.

1.6.2 Sleep Microstructure in Migraine

Patients whose attacks predominantly occur in the evening or early hours of the morning exhibit a lower cyclic alternating pattern (CAP) during NREM sleep than healthy individuals (Marca et al., 2006; Nayak et al., 2016). CAP refers to EEG activity during NREM sleep and includes transient EEG events followed by intervals of >1 minute. A high CAP during sleep is indicative of high arousal and has been linked to other sleep disorders (Parrino et al., 2012). This implies that migraineurs have a higher threshold for arousal than the general population. Sleep deprivation reduces CAP rate in healthy subjects to a rate similar to that of migraineurs baseline values (Poryazova et al., 2011). Migraine patients also showed reduced reactivity to external stimuli during NREM sleep and have less active arousal systems during REM sleep (Vollono Catello et al., 2013), and a reduction in arousal has been reported in the night preceding a migraine attack (Capuano et al., 2005; Göder et al., 2001b; Strenge et al., 2001). Such impaired arousability during sleep could be due to decreased ability to process incoming stimuli. Thus, in keeping with the idea that migraine could be in part

due to a dysfunction of sensory gating in the thalamus (see section 1.7.2 for a more detailed discussion on this; Afridi, Giffin, et al., 2005; Magon et al., 2015). However, it was reported earlier (section 1.6.1) that migraine patients with sleep-related attacks may have *more* arousals. This could reflect changes to arousal thresholds during different phases of migraine (e.g. interictal versus ictal), or alternatively could reflect hetereogenity in the timing of migraine attacks between studies (e.g. attacks during sleep, early morning), or in the measurement of arousal indices.

Related to this, there may be a relationship between sleep and cortical excitability. For example, experimental induction of CSD causes an increase in subsequent SWS duration (Cui et al., 2008), and slow wave activity in the affected hemisphere (Faraguna et al., 2010). However, it is difficult to establish cause and effect with this relationship, homeostatic sleep need may be high (e.g., due to prior sleep disruption) which then triggers CSD, or CSD could cause increased SWS as a protective response. Acute but not chronic sleep deprivation increases the frequency and lowers the threshold for CSD (Kilic et al., 2018; Negro et al., 2020). Sleep may inhibit cortical regions which have been rendered hyperexcitable in response to sensory stimuli in migraine. Although, evidence suggests that sleep spindles – a key component of NREM sleep, may facilitate cortical nociception (Claude, Chouchou, Prados, Castro, De Blay, et al., 2015), meaning that this is difficult to disentangle. In further support of this, recent studies have shown reduced sensorimotor beta power synchronisation after sleep restriction in migraine, suggesting potential alterations in GABAergic inhibitory systems, potentially driven by impaired thalamocortical connectivity (Mykland et al., 2023).

1.6.3 Sleep Disorders and Migraine

Further complicating this association, sleep disorders occur in 55% of headache patients (Ferini-Strambi et al., 2019; Paiva et al., 1997) and are more prevalent in individuals whose headaches are chronic and severe (Boardman et al., 2005; Rains, 2008a). Insomnia is the most common sleep disorder in migraine, with up to two thirds of patients reporting insomnia after controlling for anxiety and depression (Calhoun et al., 2006; Kim et al., 2017; Rains, 2008b). Treatment for insomnia using cognitive behavioural therapy results in reductions in migraine frequency (Smitherman et al., 2016), suggesting that alterations in sleep may in part underpin migraine biology.

Parasomnias (unwanted behaviours or experiences during sleep) including somnambulism (sleep walking), bruxism and restless leg syndrome are common in those with migraine (Bruni et al., 1997; Cevoli et al., 2012; Chen et al., 2010; Schürks et al., 2014). For example, the prevalence of somnambulism in migraineurs is 30–55% compared to 5–16% in controls (Montagna, 2006). Retrospective studies report higher rates of somnambulism during childhood in adult patients with migraine (Lopez et al., 2015) and children with migraine have higher rates of somnambulism than healthy control children (Barabas et al., 1983; Bruni et al., 1997; Dosi et al., 2015; Miller et al., 2003). There is little evidence linking REM-sleep behaviour disorder (RBD) to migraine, however one study found that migraine patients have a higher frequency of dream-enacting behaviour compared to controls, similar to that seen in RBD (Suzuki et al., 2013). Moreover, there is an increased rate of migraine in those with the sleep disorder narcolepsy (Dahmen et al., 2003; Suzuki et al., 2015), in which patients experience frequent and unwanted transitions into sleep during wakefulness and awaken from sleep more frequently (Sorensen et al., 2013). These clinical associations

once again highlight the complicated and multifaceted link between sleep and migraine.

1.6.4 Sleep Deprivation and Pain Perception

Aside from migraine, it is thought that sleep deprivation or disruption reduces pain thresholds and enhances pain perception (for a review see: Moldofsky, 2001). Pain and sleep have a reciprocal relationship in that sleep deprivation can cause pain and pain can cause sleep deprivation. For example, pain thresholds are reduced following REM and total sleep deprivation in animal models (Alexandre et al., 2017; Onen et al., 2001; Hicks et al., 1978) and there is increased neural activation in brain areas involved in descending modulation of pain following sleep deprivation (Kim et al., 2019).

Similar findings have been shown in humans. For example, REM sleep deprivation, total sleep deprivation, and sleep fragmentation result in reduced pain thresholds (Chiu et al., 2005; Messina et al., 2018; Smith et al., 2007) and cephalic allodynia (de Tommaso et al., 2014), with an increase proportional to the length of sleep deprivation (Azevedo et al., 2011). Furthermore, sleep loss preceding an injury can result in enhanced pain perception and increase the likelihood of chronic pain (Smith & Haythornthwaite, 2004; Vanini, 2016). Sleep deprivation can also interfere with analgesic effectiveness (Lautenbacher et al., 2006; Ukponmwan et al., 1984). These findings suggest that sleep and pain are intrinsically linked, which is likely due to their shared underlying neural networks.

1.7 Shared Neural Basis

As alluded to above, headache and sleep share a strong clinical association but are also intrinsically linked neuroanatomically and neurochemically (Holland, 2014; see **Figure 14**). Neuroimaging has revealed shared areas of activation for sleep and

headache (May et al., 1999). However, there are also intermediary regions which modulate functions such as emotion making the specific mechanisms difficult to disentangle (Brennan & Charles, 2009). The following paragraphs will briefly discuss the key regions involved in the modulation of both headache and sleep.
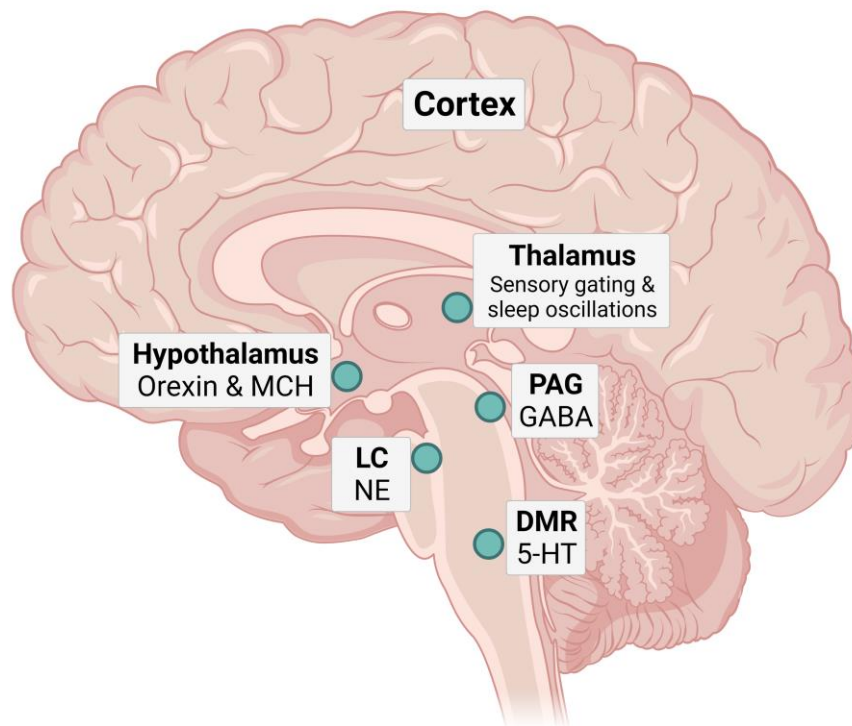
Figure 14: Shared neuroanatomical basis of sleep and migraine
Sleep and migraine are intrinsically linked neuroanatomically with shared brainstem regions (LC, DMR, PAG), diencephalic (hypothalamus, thalamus), as well as cortical. Sleep is mainly regulated by the ascending reticular activating system which involves both brainstem and diencephalic structures, with the basal forebrain and cholinergic nuclei promoting arousal and the ventrolateral preoptic hypothalamus (VLPO) promoting sleep. The noradrenergic LC and serotonergic DMR nuclei modulate arousal as well as REM sleep. The thalamus is a crucial component of sleep regulation and acts a sensory relay or gate, whose output, and thus cortical activity, depends on arousal-related inputs. The thalamus is also involved in generating sleep spindles whose function is thought to gate sensory input. There are several shared structures between sleep and headache, the most prominent of which are the PAG and the hypothalamus. The lateral hypothalamus is the primary source of the orexin peptides, which are thought to be involved in nociception. LC = locus coeruleus; PAG = periaqueductal gray, DMR: dorsal median raphe nuclei; NE = norepinephrine; MCH = melanin concentrating hormone.

1.7.1 Brainstem

The Locus Coeruleus

The LC is a small but extensively projecting nucleus located in the brainstem which plays a role in both arousal and pain modulation (Benarroch, 2018). Noradrenaline (NA; also known as norepinephrine) is the LC's principal neurotransmitter. The LC receives afferents from both the medulla and hypothalamus (Aston-Jones & Waterhouse, 2016) and projects to the reticular formation and dorsal horn of the spinal cord (Westlund & Craig, 1996). Viral tracing studies revealed two distinct subpopulations in the rodent LC, by injecting a retrograde virus into postsynaptic targets of LC neurons (Li et al., 2016). These specific sub-populations were found to have opposing antinociceptive and pronociceptive effects (Hickey et al., 2014). Furthermore, descending projections from the LC have been shown to regulate nociception at the spinal cord (Schwarz & Luo, 2015), suggesting it plays a prominent role in nociception and potentially in migraine.

The LC is active during wake and almost completely inactive during sleep (Jacobs, 1986), and is thought to play a pivotal role in the sleep/wake switch (Saper & Fuller, 2017; Takahashi et al., 2010). For example, LC NA neurons project to the VLPO area, and optogenetic activation of this circuit has been shown to mediate arousal (Liang et al., 2021). The LC is also involved in the sleep rebound as evidenced by studies in which lesioning of the LC resulted in a lack of sleep rebound in rats (González et al., 1996).

In relation to migraine, using a neurotoxin which selectively ablated NA cell populations, Vila-Pueyo et al. (2019), showed that chronic disruption of the LC inhibits trigeminal nociception, and increases susceptibility to CSD in a rat model.

Neuroimaging studies have revealed activation of the LC during evoked (Cao et al., 2002) and spontaneous migraine attacks (Weiller et al., 1995). More recent studies have revealed structural changes in the LC (Li et al., 2020), as well as altered resting state functional connectivity between the hypothalamus and the LC interictally in patients with MoA (Moulton et al., 2014). As the LC has extensive projections with diverse cortical and brainstem regions, these alterations could account for the somatosensory features associated with migraine attacks (Sprenger & Goadsby, 2009).

The Periaqueductal Gray

The PAG is a key target in migraine as it is involved in both ascending and descending modulation of nociceptive processing (Eippert & Tracey, 2014). In relation to sleep, the PAG is involved in both sleep cessation and REM-NREM transitions (Lu, Sherman, et al., 2006; Weber et al., 2018), for example it is primarily active during wake (Lu, Jhou, et al., 2006) and involved in the REM-OFF component of the flip-flop switch (see section 1.5.5). Accordingly, lesions of the PAG result in increased REM sleep (Kaur et al., 2009).

In preclinical models, electrical and pharmacological activation of the PAG is analgesic (Barbaro, 1988; Lee et al., 2000; Lee et al., 2012; Yeung et al., 1978). For example, Knight and Goadsby (2001) investigated spinal trigeminal activity in response to PAG electrical stimulation and found that trigeminal neuronal activity was inhibited by activation of the PAG, suggesting an ability of the PAG to inhibit trigeminal nociception, and this can be modulated by calcium channel blockers (Knight et al., 2003). In further support of this, lesioning of the PAG results in headache (Gee et al., 2005; Kruit et al., 2006). Conversely, stimulation of the PAG may also elicit nociceptive responses. For instance, migraine-like headaches were reported after electrical stimulation of the PAG (Raskin et al., 1987; Veloso et al., 1998).

A plethora of neuroimaging evidence has demonstrated structural (Chen et al., 2017b, 2018; DaSilva et al., 2007) as well as functional abnormalities in the PAG (Chen et al., 2017a; Gecse et al., 2022; Li et al., 2016; Mainero et al., 2011; Wang et al., 2016; Weiller et al., 1995) in patients with migraine and these alterations have been related to headache frequency (Solstrand Dahlberg et al., 2018). Additionally, evidence has revealed greater iron deposition in the PAG in patients with chronic and episodic migraine compared to healthy controls (Domínguez et al., 2019; Welch et al., 2001), and notably this is associated with poor response to treatment (Domínguez Vivero et al., 2020; Nikolova et al., 2023). These findings highlight the PAG as a major target in migraine.

1.7.2 Diencephalic

<u>The Thalamus</u>

The thalamus is a nuclear complex located within the diencephalon and its primary function is thought to be the relay, processing, and modulation of sensory and motor information from the periphery to the cortex (Hwang et al., 2017). The thalamus also plays an important role in cortico-cortical communication (Guillery, 1995) and modulating emotion and cognition (Wolff & Vann, 2019).

As well as modulating sensory and nociceptive input, the thalamus has an important role in sleep (Gent et al., 2018). During wake thalamic neurons are depolarised and thalamocortical circuits are activated facilitating the transmission of sensory information to higher cortical structures (Weyand et al., 2001). As the brain transitions to SWS, these neurons become hyperpolarised, and exhibit burst patterns which gate sensory transmission (Yamadori, 1971). Such sensory dissociation may be involved in ensuring sleep stability (Dang-Vu et al., 2010). Furthermore, thalamic

reticular GABAergic neurons are involved in generating sleep spindles (Bastuji et al., 2020). Spindles are thought to inhibit ascending information from reaching the cortex which may help to gate nociceptive responses from disturbing sleep. Although this has recently been contested, with studies showing no inhibitory effect of spindles on sensory or nociceptive processing (Claude, Chouchou, Prados, Castro, De Blay, et al., 2015; Claude et al., 2016).

The accompanying sensory disturbances during migraine implicate the thalamus as a key hub in migraine pathophysiology (Younis et al., 2019). Indeed, there is thalamic activation following activation of the trigeminal system (Burstein et al., 2010) and the familial hemiplegic migraine (FHM1) mouse shows altered thalamic responses to TG activation (Park et al., 2014). Neuroimaging studies have observed structural (Granziera et al., 2014) and functional changes (Coppola et al., 2016; Wang et al., 2016; Xue et al., 2013) in the thalamus in migraine patients. Furthermore, in patients who experience allodynia during attacks, the posterior thalamus is hyper-responsive when the patient is presented with thermal stimuli (Maleki et al., 2021). Although the clinical significance of these findings remains unclear.

Given the intricate role of the thalamus in the regulation of sleep and pain, it is likely that thalamic mechanisms may in part underlie the clinical association between sleep and migraine. For example, it is considered that migraine is a disorder of hyperexcitability or an imbalance in hyper and hypo-excitability (Gollion, 2021). Thus, alterations in thalamic sleep spindles or thalamocortical circuits that modulate sensory processing may predispose an individual to migraine triggering.

<u>The Hypothalamus</u>

Preclinical and clinical studies have identified the hypothalamus as a key target in both pain and primary headaches (Inutsuka et al., 2016; Karsan & Goadsby, 2020)., and it is considered as a potential candidate for attack initiation (Schulte et al., 2017). As well as forming a major part of the sleep/wake flip-flop switch and sleep initiation (see **Figure 14** above), the hypothalamus plays an integrative and regulatory role in autonomic, homeostatic, cognitive, and endocrine functions (Montagna, 2006; Saper, 2002). Moreover, the premonitory symptoms associated with migraine such as food cravings, mood changes and thirst (Maniyar et al., 2014) and the fact that migraine can be triggered by external and internal changes to the body (e.g. menstruation, weather, physical activity, jet lag; Andress-Rothrock et al., 2010) imply that altered homeostatic control may underlie the shared aetiology of sleep and migraine. Related to this, headache disorders exhibit a clear seasonal and circadian rhythmicity (Naber et al., 2019; Zurak, 1997), highlighting the hypothalamus and SCN as a likely neural hub in the pathophysiology of headache.

Further, the hypothalamus is linked anatomically with pain-related nuclei such as the LC, PAG, and DMR which are also involved in sleep regulation (Robert et al., 2013). The LH is the primary source of the orexin peptides which are thought to be involved in both nociception and sleep (Holland & Goadsby, 2007; Holland, 2017) and the LH has diverse projections across the pain neuroaxis (cortex, thalamus, amygdala, dorsal horn of the spinal cord) (Chiou et al., 2010). Furthermore, hypothalamic activation has been shown in response to superior sagittal sinus (SSS) and dural stimulation as assessed by up-regulation of c-Fos immunoreactivity in the posterior hypothalamus (Benjamin et al., 2004; Malick et al., 2001), demonstrating the importance of the hypothalamus in trigeminal nociception.

Neuroimaging studies have observed greater Blood Oxygenation Level Dependent (BOLD) and metabolic responses in the hypothalamus during the premonitory phases and during attacks, and altered functional coupling with spinal trigeminal nuclei (**Figure 15**; Denuelle et al., 2007; Schulte & May, 2016). Recent data revealed altered BOLD response to oral glucose in the hypothalamus in migraine patients when administered the clinical migraine trigger nitroglycerin suggesting that this activation is not limited to spontaneous attacks (van Oosterhout et al., 2021). Moreover, an abnormal circadian hypothalamic hormonal profile has been shown in patients with chronic migraine (Peres et al., 2001). Taken together, these findings suggest that the hypothalamus is an important intersection between sleep physiology and headache pathophysiology; and may be a key target for migraine.
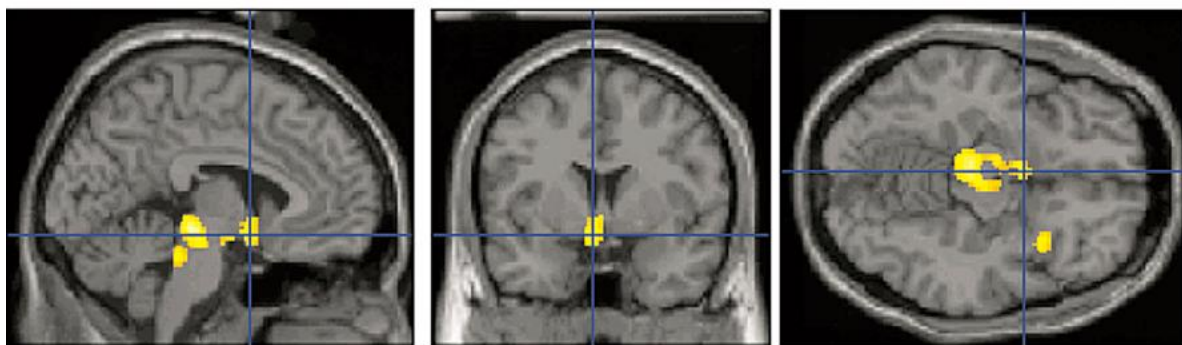
Figure 15: Hypothalamic activation during spontaneous migraine attacks
Figure taken from Denuelle et al. (2007). This shows the areas of significant regional cerebral blood flow increases (yellow) superimposed on a T1-weighted MRI anatomical reference, highlighting an increase in the hypothalamus.

1.7.3 Neuropharmacology

Migraine and sleep share many of the same neurotransmitters and neuropharmacology. The following section will review several key neurotransmitters and neuropeptides likely involved in the shared aetiology.

Orexins

The orexin system is comprised of two G-protein coupled receptors (GPCR): orexin-1 (OX_1R) and orexin-2 (OX_2R) (see **Figure 16**). There are two neuropeptides orexin-A (OXA) and orexin-B (OXB) which are cleaved from the precursor protein prepro-orexin (Sakurai et al., 1999). Whilst activation of both receptors is excitatory, the OX_1R is selective for OXA, whereas the OX_2R is non-selective for OXA and OXB (Kim et al., 2004). However, OXB has a 10-fold higher affinity for the OX_2R than OX_1R (Heifetz et al., 2013). As mentioned above, the orexin peptides are secreted from approximately 70,000 orexin-producing neuronal cell bodies (20% of LH neurons) which are located exclusively in the LH, yet orexinergic neurons exhibit diffuse projections across the CNS, whereby they have excitatory influences on arousal centres (Thannickal et al., 2009). Indeed, orexinergic fibres project to the LC, thalamus and PAG with direct projections from the LH to lamina I of the spinal cord; and orexin receptors are found in the dorsal root ganglion implicating the orexins in nociceptive processing (Bingham et al., 2001; Giesler, 1995; Pol, 1999). Orexinergic neurons also receive nociceptive input from the dorsal horn of the spinal cord via the lateral parabrachial nucleus (Asano et al., 2019). However, as well as nociception, orexinergic neurons project to a variety of regions involved in various functions (see **Figure 17**) including appetite regulation (Sakurai, 1999), respiration (Nattie & Li, 2012), and reward (Aston-Jones et al., 2009).
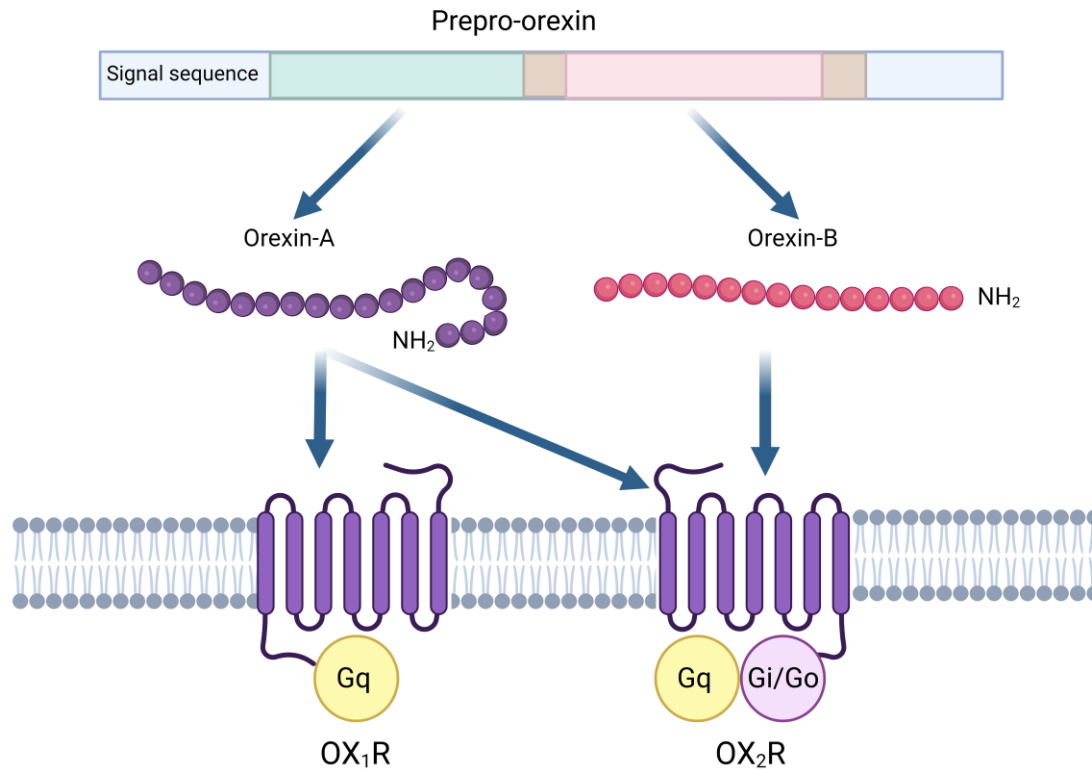
Figure 16: Schematic representation of the orexinergic system
The orexin system is comprised of two G-protein coupled receptors (GPCR): orexin-1 (OX_1R) and orexin-2 (OX_2R). Two neuropeptides: orexin-A and orexin-B are cleaved from the precursor protein prepro-orexin (Sakurai et al., 1999). The OX_1R is selective for OXA, whereas OX_2R is non-selective for OXA and OXB. However, OXB has a 10-fold higher affinity for the OX_2R than the OX_1R (Kim et al., 2004). The OX_1R couples to the G_q class of G protein, whereas OX_2R couples with G_i or G_o.
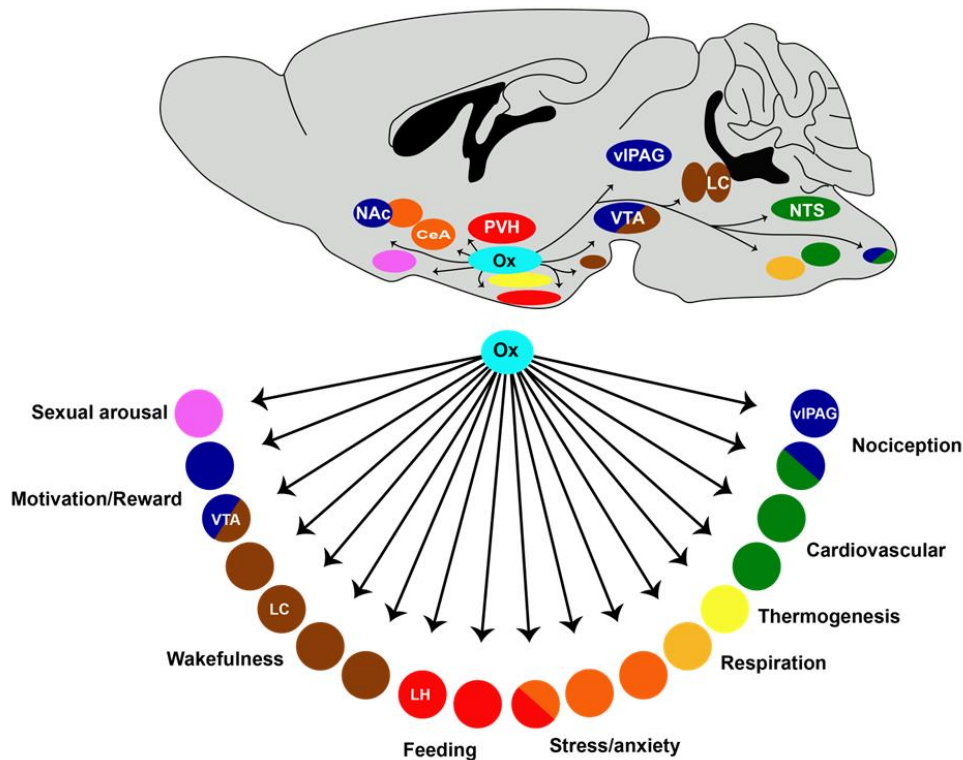
Figure 17: Orexinergic projections and their proposed functions
Taken from Sureda-Gibert (2022). The orexins are exclusively synthesized in the lateral hypothalamus (LH) and project to many areas of the CNS including brainstem and cortical regions. The orexins are proposed to be involved in a variety of functions from reward, feeding, cognition, to nociception.

The Orexins and Sleep

As mentioned in section 1.5.2 the orexins play a key role in stabilising the waking state. Initial studies demonstrated that electrical stimulation of the LH led to increased wakefulness, and lesions of the LH resulted in sleep (Gerashchenko et al., 2001; Levitt & Teitelbaum, 1975). Moreover, discovery of the orexins' involvement in sleep/wake regulation came from studies of canine narcolepsy (Lin et al., 1999). Narcolepsy is a sleep disorder characterised by an 80-90% reduction in orexinergic neurons (Thannickal et al., 2000), and patients with narcolepsy with cataplexy – narcolepsy type 1 (NT1), demonstrate decreased levels of OXA in CSF (Peyron et al., 2000).

In healthy individuals, orexin levels in the hypothalamus exhibit diurnal variation and are maximal during wake (Kiyashchenko et al., 2002; Taheri et al., 2000)

and after sleep deprivation (Estabrooke et al., 2001; Modirrousta et al., 2005). Orexinergic neurons inhibit sleep promoting regions including the VLPO to induce wakefulness (De Luca et al., 2022), and conversely GABA-ergic neurons in the VLPO innervate the LH to promote sleep (Matsuki et al., 2015). Direct injection of OXA during the light period (sleep) in rats results in an increase in wakefulness (Hagan et al., 1999), again highlighting their function as a wake-promoting or wake-stabilising neuropeptide.

The orexins are also specifically involved in gating REM sleep. The most obvious example being orexin-deficient narcolepsy, in which patients present with abrupt transitions into REM upon sleep onset – sleep-onset REM periods (SOREMP; occurrence of REM sleep within 15 minutes of sleep onset), as well as frequent transitions between NREM and REM sleep (Kornum et al., 2017; Pizza et al., 2015; Sorensen et al., 2013). This is further supported by the finding that patients with NT1 often have co-morbid RBD, which is also attributed to the loss of orexin (Knudsen et al., 2010). The OX_1R is thought to be primarily responsible for these alterations in REM sleep as OX_2R-knockout mice are only display a mild SOREMP phenotype (Willie et al., 2003). In line with this, orexinergic neurons have been shown to increase their firing just before the end of REM sleep and thereby "herald" the transitions to wake (Lee et al., 2005), lending further support for their role in sleep-wake and sleep-stage transitions.

The Orexins and Pain

Destruction of the posterior hypothalamus has been shown to result in transient hyperalgesia (Millan et al., 1983), and OXA has been shown to have antinociceptive effects in various pain models (Holland et al., 2005; Razavi & Hosseinzadeh, 2017; Suyama et al., 2004; Yamamoto et al., 2003). For example, in animal models,

administration of OXA inhibits freezing behaviour, photophobia, formalin-induced inflammatory pain, and thermal hyperalgesia (Haghparast et al., 2020; Kajiyama et al., 2005; Kooshki et al., 2020; Yamamoto et al., 2002). Furthermore, OXA can recover incision-induced hind-paw allodynia (Cheng et al., 2003; Yamamoto, Saito, Shono, Aoe, et al., 2003), and OXA modulated dural nociceptive input to the TNC when injected into the posterior hypothalamus (Bartsch et al., 2004) and PAG (Holland et al., 2006). Conversely, OXB was found to be pro-nociceptive (Bartsch et al., 2004). OXA inhibited electrically-induced vasodilation in an animal model and this was reversed by pre-treatment with an OX_1R antagonist SB-334867 (Holland et al., 2005), whereas OXB had no significant effect.

The Orexins and Migraine

The orexins are hypothesised to play a role in migraine (Holland & Goadsby, 2007). Narcoleptic patients report increased frequency of migraine (Dahmen et al., 2003). Similarly, people with migraine are more likely to develop narcolepsy than healthy controls (Yang et al., 2017) suggesting a shared pathophysiology related to the orexinergic system.

Therapeutics targeting the orexin system have shown promise in treating migraine (Hoffmann, Supronsinchai, et al., 2015; Strother et al., 2018). For example, dual orexin-receptor antagonists (DORAs) were developed for insomnia and have proven efficacy (Coleman et al., 2011; Xue et al., 2022). In preclinical models, a precursor of suvorexant (DORA-12) was shown to inhibit trigeminal nociception in response to electrical stimulation of dural trigeminal afferents and increased the threshold for inducing CSD (Hoffmann, Supronsinchai, et al., 2015). However, a recent clinical trial with the DORA Filorexant found no significant reduction in monthly migraine days (Chabi et al., 2015). Although, this finding could be the result of a variety

of factors. For example, Filorexant was given at night when endogenous orexin levels are already low (Grady et al., 2006). It is possible that orexin antagonists delivered at other circadian times could be beneficial. Delivery could be tailored to different individual chronotypes and endogenous circadian periods, especially given the symptoms and severity of insomnia (which DORAs were originally developed for) differ depending on chronotype (Ong et al., 2007). Chronotherapy studies (Cardinali et al., 2021) should be conducted to establish this. Indeed, orexin and peptides involved in headache such as CGRP show clear diurnal variation (Ashina, Bendtsen, Jensen, Schifter, Jansen–Olesen, et al., 2000; Grady et al., 2006; Wimalawansa, 1991), thus further research investigating the temporal dynamics of orexin's anti-nociceptive properties is warranted. Moreover, DORAs as the name suggests, antagonise both orexin receptors, whereas OXA which preferentially binds to the OX_1R has been shown to be anti-nociceptive whereas OXB has been shown to be pro-nociceptive.

Serotonin

Serotonin (5-hydroxytryptamine; 5-HT) is primarily an inhibitory neurotransmitter synthesized from the essential amino acid tryptophan (Clark et al., 1954). Its most prominent expression is within the DRN neurons in the brainstem but is widely distributed throughout the brain (Mohammad-Zadeh et al., 2008).

Early studies on sleep regulation showed that pharmacological inhibition of 5-HT resulted in severe insomnia in cats (Denoyer et al., 1989), and therefore 5-HT was postulated to be a sleep-promoting neurotransmitter. However, evidence challenges this and 5-HT is thought to be involved in both the inhibition and facilitation of sleep (Jouvet, 1999; Tricklebank, 2019; Ursin, 2002). For example, neurons in the sleep-promoting VLPO are either inhibited or activated by 5-HT (Gallopin et al., 2000, 2005; Rancillac, 2016). 5-HT neurons fire predominantly during wake, decrease during

NREM sleep and are largely silent during REM sleep (Venner et al., 2020), implicating 5-HT as a REM-OFF modulator (Lu, Sherman, et al., 2006; McGinty, 2009), as well as being involved in arousal.

5-HT has been intrinsically linked to the pathophysiology of migraine (Hamel & Currents, 2007; Silberstein, 1994) and plays a key role in nociceptive processing (Martins, 2019). Migraineurs have been reported to have both higher (Deen et al., 2018; Sicuteri et al., 1961) and lower (Ferrari & Saxena, 1993) plasma or cerebral 5-HT levels interictally, and elevated levels during attacks (Deen et al., 2017). However, some studies report no difference in serotonin levels (Jernej et al., 2002). Pharmacological depletion of tryptophan levels was shown to exacerbate migraine-related symptoms such as photophobia and nausea in migraine patients and healthy controls (Drummond, 2006), and low 5-HT levels have been shown to be related to lower pain thresholds (Schwedt, 2007). Additional evidence for a role of serotonin is that $5\text{-HT}_{1B/1D}$ receptor agonists – triptans, and 5-HT_{1F} receptor agonists - ditans serve as acute therapy for migraine attacks (Negro et al., 2018); and regulate trigeminal sensory activation (Shields & Goadsby, 2006). These observations support a role for altered serotonergic transmission in migraine pathophysiology.

<u>Adenosine</u>

Adenosine is involved in the initiation of sleep (see section 1.5.1). It accumulates throughout the day particularly in the basal forebrain (Peng et al., 2020). Adenosine promotes sleep via inhibitory A_1 receptors and inhibits arousal promoting regions (orexinergic and cholinergic neurons) via A_{2A} receptors (Porkka-Heiskanen et al., 1997). The action of adenosine over 5-HT is thought to be responsible for thalamic sensory gating during sleep (Yang et al., 2015). Adenosine had excitatory influences on the sleep-promoting VLPO area (Chamberlin et al., 2003). Caffeine – the well-known

stimulant, combats subjective sleepiness by antagonising both the adenosine A_1 and A_{2A} receptor subtype (Fredholm, 1995; Reichert et al., 2022).

In preclinical models, direct administration of adenosine has been shown to have antinociceptive properties at the dorsal horn of the spinal cord (Yoon et al., 2005), and A_1 receptor agonists inhibit trigeminovascular afferents (Goadsby, Hoskin, et al., 2002). Adenosine A_1 receptor activation in peripheral nerve endings prevents hind-paw mechanical allodynia in rats (Taiwo & Levine, 1990). Moreover, adenosine A_1 receptor knock-out mice exhibit increased thermal algesia, and do not show typical analgesic effects of adenosine administration (Gong et al., 2010).

Clinically, the role of adenosine in headache is complex (Nowaczewska et al., 2020; Thuraiaiyah et al., 2022). Caffeine is often used as an analgesic to treat headache (Lipton et al., 2017), but is also implicated as a precipitating factor (Scher et al., 2004) and leads to migraine chronification. However, this is most likely related to withdrawal from caffeine, rather than acute use (Alstadhaug et al., 2020). Moreover, chronic caffeine use has complex effects on sleep (O'Callaghan et al., 2018; Panagiotou et al., 2019) increasing sleep need and NREM duration, further confounding the relationship between adenosine and headache. Specifically in relation to migraine, circulating serum adenosine levels have been shown to be higher in MoA during attacks (Guieu et al., 1994, 1998), and administration of adenosine can trigger migraine attacks in migraine patients and migraine-free individuals (Birk et al., 2005; Brown & Waterer, 1995; Kruuse et al., 2000, 2006). Taken together, this suggests a multifaceted relationship between adenosine, sleep, and migraine.

Melatonin

Melatonin (N-acetyl-5-methoxytryptamine) is secreted mainly from the pineal gland and is often regarded as the "dark hormone" being tightly regulated by light levels. The greatest levels are observed during the night and the lowest levels in the morning (Wurtman et al., 1963). Endogenous melatonin secretion is regulated by the SCN and strongly entrained to the light-dark cycle (Claustrat et al., 2005), and the onset of melatonin coincides with the increase in nocturnal sleepiness (Cajochen et al., 2003). Melatonin acts primarily via membrane GPCRs MT_1 and MT_2 (Pandi-Perumal et al., 2008). Endogenous melatonin is derived from serotonin (Thor et al., 2007), therefore treatments with a serotonergic effect (e.g., triptans) may modulate melatonin secretion which could impact sleep. This could be either by alteration of the amplitude of melatonin secretion (and thus affecting the circadian drive to initiate sleep), or by modulating sleep continuity (Nesbitt et al., 2014).

In preclinical models, melatonin is shown to be anti-nociceptive (Srinivasan et al., 2012). Ablation of the pineal glands in rats results in enhanced trigeminovascular activation which may be normalised by administration of melatonin (Tanuri et al., 2009), and melatonin reduces formalin-induced nociception and allodynia in rats (Arreola-Espino et al., 2007). Additionally, melatonin pre-treatment significantly reduced CSD-evoked micro neurovascular changes and associated trigeminal nociception as assessed by c-Fos activation in the TNC (le Grand et al., 2006).

In migraine patients, attenuated melatonin secretion has been observed in urine (Brun et al., 1995; Kozak et al., 2017; Masruha et al., 2008; Peres et al., 2001; Toglia, 1986), and serum (Liampas et al., 2020) with clear sex differences in that females show a greater decrease in melatonin compared to males, and increased corresponding light sensitivity (Claustrat et al., 1989, 2004). However, some studies report no differences

interictally, with some differences being reported dependent on the duration of the migraine attack (Zduńska et al., 2021). Moreover, melatonin-based therapies have proven to have modest efficacy in treating migraine (Eli & Fasciano, 2006; Gagnier, 2001; Long et al., 2019), highlighting further the link between sleep and migraine.

1.8 Hypothesis and Aims

Due to the striking overlap between migraine and sleep at the clinical, neuroanatomical, and molecular level we hypothesise that dysregulation of underlying neural networks regulating sleep-wake cycles results in enhanced pain perception and contributes to migraine attack initiation and susceptibility. We aimed to investigate this hypothesis in three ways:

1) Although there is an association between sleep and migraine, it remains unclear whether patients suffer from altered sleep compared to migraine-free individuals. Through human data, we aimed to disentangle this relationship and establish whether patients exhibit alterations in sleep (subjectively and objectively), and whether sleep disruption may predict migraine attacks.

2) Secondly, to directly assess the influence of sleep deprivation on migraine, we sought to use mouse models of sleep deprivation and assess the impact on translational migraine-related readouts. We also aimed to explore potential mechanisms and therapeutic interventions for migraine.

3) Lastly, given the potential role of sleep deprivation in migraine susceptibility, and the proposed relative resistance of FNSS individuals to life stressors and pain, we used a novel transgenic mouse line harboring a mutation for FNSS and sought to determine whether they exhibit reduced migraine susceptibility.

Chapter 2: Preclinical Methods

2.1 Sample Size Calculations

Sample size estimation for each animal experiment was calculated using G*Power using the following formula (Faul et al., 2007):

$$n = f(\alpha, \beta) \left[\frac{2SD^2}{difference\ in\ means^2} \right]$$

The sample size for mechanical withdrawal threshold assessment was based on previous pilot data and published studies (Moye & Pradhan, 2017): effect size = 1.5, α = 0.050 and power = 0.80, therefore an adequately powered behavioural study requires at least n = 8 mice per group. This number was deemed appropriate for tissue and biochemical analysis. For electrophysiology, sample size was determined using our published data (Holland et al., 2012) where difference in means = 24%, standard deviation (SD) = 18-22% and thus an adequately powered study requires n = 10-12 per group. For circadian rhythm analysis, the sample size was based on previous data from our laboratory (Strother, 2020) where difference in means = 6%, effect size = 1.3, power = 0.80, an adequately powered study requires a sample size of at least n = 2 per group.

2.2 Animals

All animal procedures in this thesis were conducted in strict accordance with the United Kingdom Home Office Animal (Scientific Procedures) Act (1986), under project licences P620D39BA and PP9890223, and in agreement with Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines (Percie du Sert et al., 2020). All experimental procedures were approved by the King's College London Animal Welfare and Ethical Review Body (AWERB). Mice of a C57Bl/6J genetic background, both males and females were used in all experiments and purchased from Charles River, United Kingdom or bred in-house. Unless otherwise stated, mice were group

housed within the Biological Services Unit (BSU) in a climate-controlled room on a 12-hour light/dark cycle with access to food and water ad libitum. The experimenter was always blinded to experimental conditions and animal genotype during testing and analysis.

2.2.1 Breeding and Genotyping of Transgenic Mice

Transgenic human Differentiated Embryo Chondrocyte 2 (DEC2) proline to arginine at the 384aa and FLAG tag (P384R-flag) familial natural short sleeper (FNSS) mice were created using human Bacterial Artificial Chromosome (BAC) microinjection random insertion by Professor Louis Ptáček at the University of California San Francisco (He et al., 2009a) previously incorrectly reported as the P385R mutation in the published article). These mice were kindly provided for use in this project by Professor Louis Ptáček. These transgenic mice need to conditionally express both human wild-type (WT) (*h*DEC2WT-BHLHE41-1Yfu) (N10)- MBP-1049) and human mutant DEC2 (*h*DEC2-P384R). Random insertion results in 1-2 copies of the P384R mutation. Transgenic hemizygous mice with one- two copies of the mutant *h*DEC2 and one copy of the WT *h*DEC2 will be referred to as *h*DEC2-P384R hereafter.

To establish whether any phenotype is independent of the extra copies of the transgene being inserted into the genome, or due to over-expression of certain genes, a line containing the WT human DEC2 transgene (*h*DEC2WT-BHLHE41) in addition to the endogenous DEC2 mouse alleles is required as a 'dosage control'. This strain will be referred to as DEC2-WT and will be used as a control line (see **Figure 18**). Each line produced litters of approximately 50% WT and 50% hemizygotes. Mice were bred WT × hemizygote and maintained at King's College London in the BSU and the genotypes of offspring were determined by in-house quantitative polymerase chain

reaction (qPCR) as described in the next section. Both the hemizygous (hemi) mice were used along with their WT littermates as controls.

Murine DEC2 is a transcriptional repressor which belongs to the basic helix-loop-helix family. It is a negative component of the circadian clock and represses CLOCK/BMAL1-induced transactivation through E-box elements (see **Figure 6**). The P384R mutation changes a C to a G in the DEC2 DNA sequence which is proposed to cause a proline-to-arginine alteration at amino acid position 384. This mutation results in a reduction of sleep during the light phase, and a shortened overall activity period and a reduced sleep rebound (He et al., 2009).
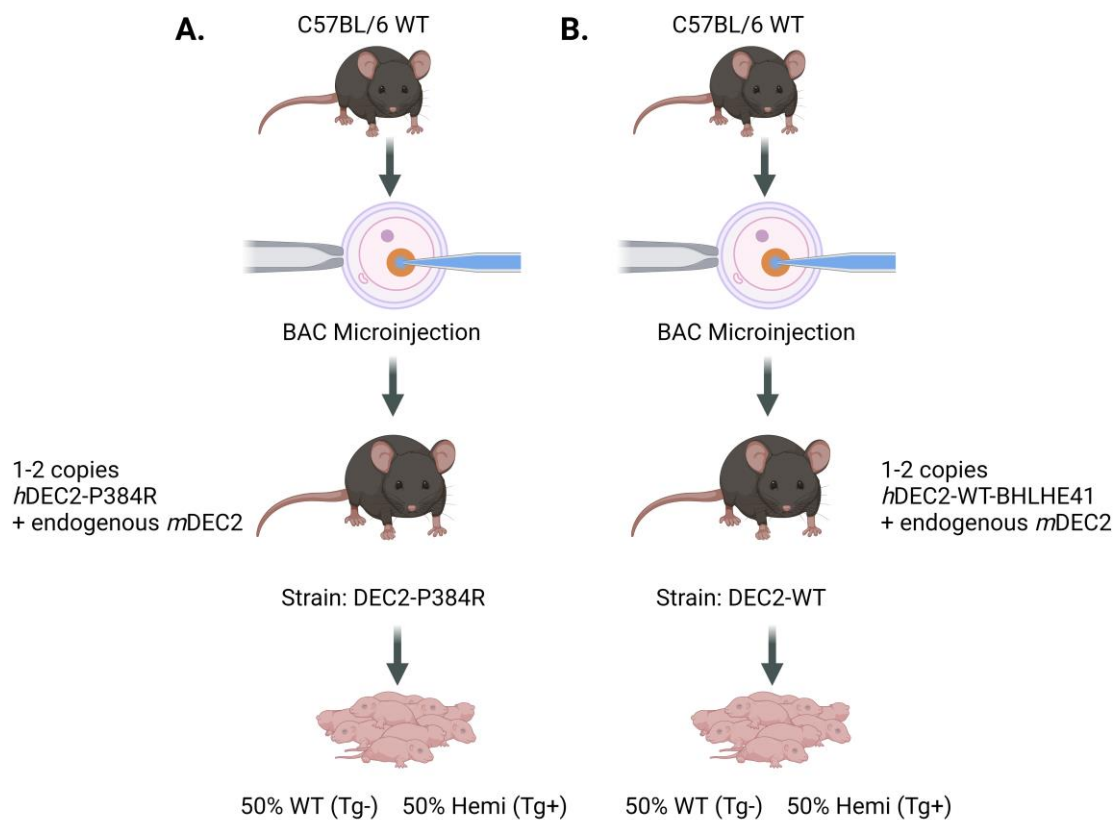
Figure 18: Generation of transgenic *h*DEC2-P384R and *h*DEC2-WT mice

Both strains were created by bacterial artificial chromosome (BAC) microinjection by Professor Ptáček on a C57/Bl/6J background. *h*DEC2-P384R have 1-2 copies of the P384R mutation randomly inserted into the mouse genome, whereas *h*DEC2-WT have 1-2 copies of the wild-type (WT) *h*DEC2 allele. In both cases, litters were made up of approximately 50% WTs and 50% hemizygotes (hemi).

2.2.2 Deoxyribonucleic acid (DNA) extraction

2-10 mg of ear tissue was taken with an ear punch which was sterilized with 70% ethanol between each mouse and stored in 0.5 ml Eppendorf tubes. To extract DNA, the "HotSHOT" method was followed (Truett et al., 2000) whereby 75 µl of alkaline lysis buffer (99 ml H_2O, 250 µl of 10N sodium hydroxide (NaOH), 40 µl of ethylenediaminetetraacetic acid (EDTA) solution), was added to the ear tissue. The solution was incubated at 95°C for 30 minutes, then 75 µl of neutralizing buffer (96 ml H_2O and 4 ml 1M Tris-Hydrochloric acid was added. The solution was mixed and centrifuged for two minutes at 15,000 rotations per minute (rpm) before being used in the genotyping assay. Digested DNA was stored at -20°C for later use.

2.2.3 Quantitative Polymerase Chain Reaction (qPCR)

To identify the genotypes of offspring, Quantitative Polymerase Chain Reaction (qPCR) was used. The reaction contained: 5 µl of Express SYBR Green (Invitrogen, A1031), 4 µl of RNAse-free H2O, 0.6 µl primer mix, and 0.4 µl of extracted DNA for a total reaction volume of 10 µl per well. Forward and reverse oligonucleotide primers (5µM concentration per primer) (Merck Life Science UK Ltd) were designed and validated by Professor Louis Ptáček's team at the University of California San Francisco. Casein kinase 1 epsilon (CK1ε) was chosen as the internal control as it is also a component of the molecular circadian clock (Takano et al., 2004) and does not show oscillatory expression (Ko & Takahashi, 2006) and thus should always be present. The oligonucleotide sequence and product size for each primer is shown in **Table 3**. The final reaction solution was added to wells of a 96-well opaque microplate which was sealed with a plate sealer and centrifuged in a plate centrifuge (Multifuge™, Thermo Fischer) for 2 minutes, at 4°C at 5,000 rpm. The plate was processed in a thermocycler (Roche LightCycler® 408), following the program shown in **Table 4**.

Table 3: Primers used in the qPCR melt curve genotyping

Primer Name	Primer Sequence	Fragment Size (bp)
	*h*DEC2	
*h*DEC2 Forward	5' - AAGTTCCTGACTTAAAACTTGTGCTATACCG - 3'	118
*h*DEC2 Reverse	5' - ACTTCTTGAGCTTTCCACAAGACAGGTTAT - 3'	
	CK1ε	
Ck1ε Forward	5' - GCCGTCGAGATGACCTAGAG - 3'	383
Ck1ε Reverse	5' - GCTTCCATTTCCTCAAACC - 3'	

Abbreviations: bp = base pairs, qPCR = quantitative polymerase chain reaction

Table 4: Thermocycler conditions

Step	Temperature	Time (s)		Ramp Rate (°C/s)
Hold Stage	95°C	60		1.60
PCR Stage	95°C	15	Repeated x35	1.60
	60°C	30		1.60
Melting Curve	95°C	15		1.60
	60°C	60		1.60
	99°C	Continuous		0.11
Cooling	40°C	30		2.20

PCR = polymerase chain reaction

2.2.4 Melt Curve Genotyping

To determine the genotype of the genomic DNA (gDNA) sample, a melt curve analysis was conducted. This method allows you to distinguish the qPCR products based on the amplicon melting and resulting fluorescent intensity (Papp et al., 2003). Each allele has a different melting temperature and a resultant melting curve which can be visualised. See **Figure 19** for a typical melt curve analysis output of DNA samples. In this case we only probed for the presence of the human DEC2 allele (WT or mutant). To determine whether a mouse has the mutant or WT version of *h*DEC2, we simply need to be aware of the transgenic line from which it was generated. Notably, this approach does not distinguish hemizygous from homozygous animals.
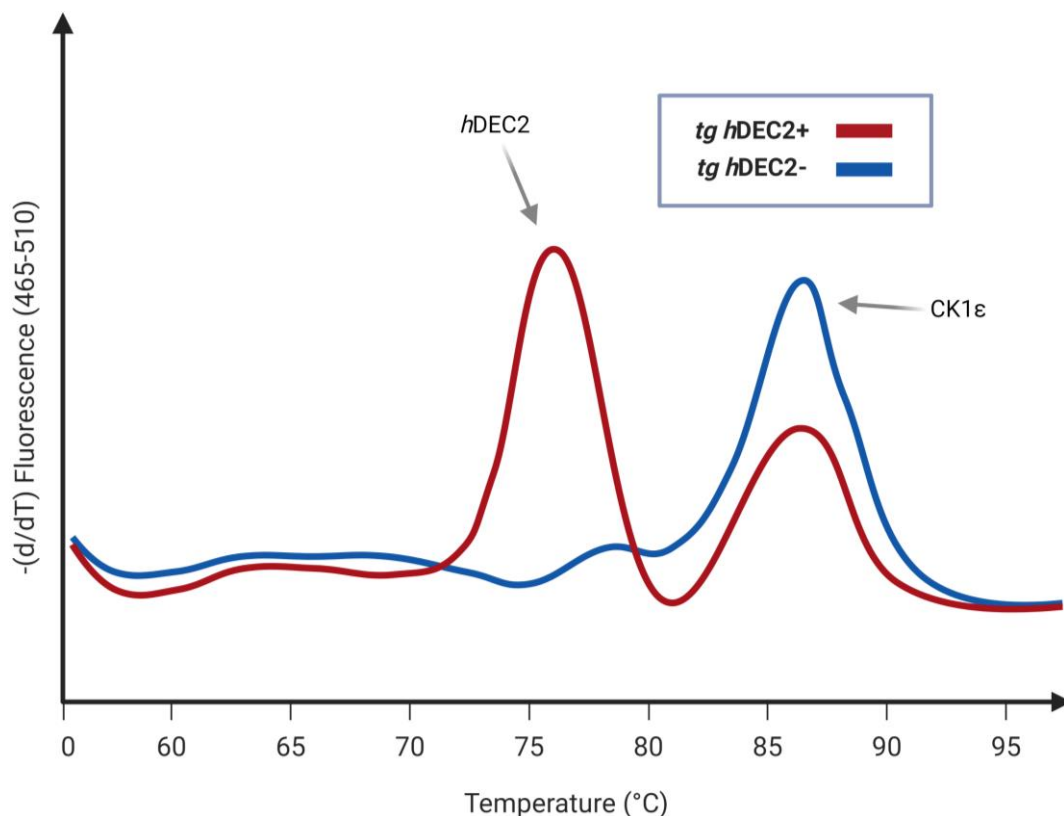
Figure 19: Schematic of melt curve genotyping results
The genotyping results splits the sample into two groups based on the melting point of the DNA fragments. Casein kinase 1 epsilon (Ck1ε) was used as the internal control. The largest red peak indicates hemizygous mice which have 1-2 copies of the *h*DEC2 allele (mutant or WT dependent on the strain; Transgenic/Tg+), as well as the CK1ε control, whereas the blue group indicates wild type mice with no human DEC2 allele present (Tg-). *h*DEC2 transgene melting temperature = 75-80°C. Ck1ε transgene melting temperature = 89°C.

2.3 Electrophysiology

2.3.1 Cortical Spreading Depression

Anaesthesia and surgical procedures

Anaesthesia was induced using Isoflurane (4% induction, 1.5-2% maintenance). Depth of anaesthesia was continuously monitored and assessed by the absence of the paw pinch reflex. Body temperature was maintained with a homoeothermic blanket (Harvard Apparatus™). Eye gel was applied to each eye throughout surgery to prevent corneal drying (Viscotears, Alcon, UK). The mouse was placed in a stereotaxic frame and a medial incision was made to expose the surface of the skull and a cranial window was drilled between lambda and bregma to expose the underlying cortex. Mineral oil was applied to the cortical surface to prevent drying. All drilling was conducted using a saline-cool drill to prevent overheating and damage to the cortex. A small hole was made in the left hemisphere anterior to bregma with a 23-gauge needle for recording (see **Figure 20** for the placement of electrodes). A glass capillary (tip diameter 10 μm) filled with 3M NaCl was placed in the hole 200 μm below the cortical surface for recording of cortical steady state potential (Direct current - DC shift). The glass capillary was coupled to a silver/silver chloride (Ag/AgCl) pellet and an Ag/AgCl reference electrode (World Precision Instruments) was placed subcutaneously in the nuchal muscle. The electrode was connected to a high-impedance head stage and the signal was fed through a DC preamplifier (Neurolog NL102, gain x 1000), filtered (N125), then passed through a second stage amplifier (NL106), through an analogue-to-digital converter (Power 1401plus, CED, UK) and displayed on a personal computer (Spike5, CED, UK).

Cortical spreading depression induction and recording

Before any recording took place there was a 30-minute rest period to allow for the signal to stabilise. CSDs were induced by placing a cotton pellet soaked in 1M Potassium Chloride (KCl) over the cranial window posterior to the recording electrode. Subsequently, 5 μl of KCl was applied to the cotton pellet every 15 minutes with a pipette and the number of CSDs were counted over a total recording period of 1 hour (4 KCl applications). Animals were then terminated via cervical dislocation.
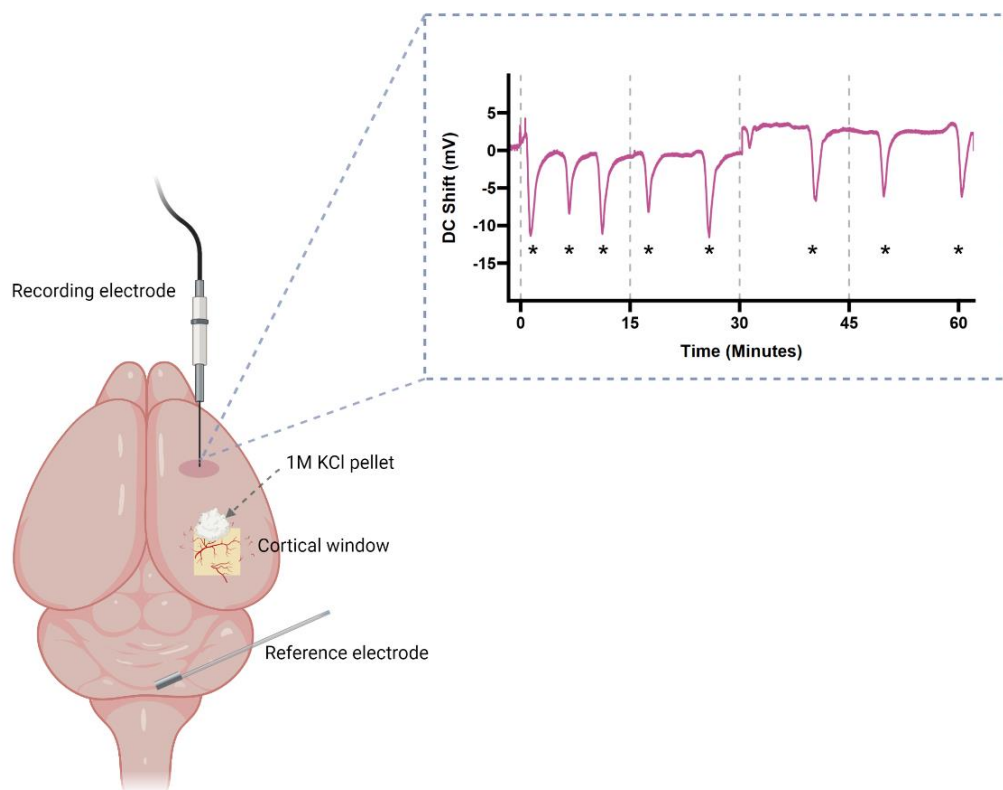
Figure 20: Recording setup for induction of cortical spreading depression
Schematic of the CSD recording setup. In anesthetized mice a craniotomy was drilled to create a cortical window. A glass recording electrode filled with 1M NaCl was inserted approximately 200 μm under the cortical surface to record the direct current (DC) shift, with a reference electrode placed in the nuchal muscle. A cotton pellet soaked in 1M KCl was placed on the induction site for CSD initiation. The windows shows a representative electrophysiological trace of DC potential highlighting a shift of more than 5 mV change from baseline representative of a CSD event (*). KCl = potassium chloride.

The number of CSDs were counted manually by visual inspection of the electrophysiological trace. This enabled standardization of counting across animals. We adhered to a threshold of 5 mV change from baseline for confirmation of a CSD (Vila-Pueyo et al., 2019). The number of CSDs over 1 hour of recording was used as a measure of CSD susceptibility and was compared between transgenic mice and their WT littermates.

2.3.2 Electroencephalography

Anaesthesia and Surgical procedure

Mice were implanted with EEG and EMG electrodes for monitoring brain and muscle activity for sleep analysis under Isoflurane anaesthesia (3.5% induction, 2-2.5% maintenance). Depth of anaesthesia was continuously monitored and assessed by the absence of the paw pinch reflex. Body temperature was maintained with a homoeothermic blanket (Harvard Instruments, UK). Eye drops were applied to each eye throughout surgery to prevent drying (Lacrilube®, Allergan Ltd., UK). A small incision was made on the scalp, exposing the skull which was cleaned with saline followed by 3% Hydrogen Peroxide (H_2O_2) to aid visualisation of bregma and lambda. A head-mounted preamplifier (Pinnacle Technology, Inc.) was affixed over bregma using four 1.6 mm stainless steel mounting screws (Bilaney Consultants Ltd.) (10-50 kΩ), including a common reference and ground (see **Figure 21** for electrode placement).

The first EEG channel was placed in the left medial frontal lobe (+2.16 mm anterior-posterior (AP), +2.75 medial-lateral (ML) from bregma) the second was placed in the right primary somatosensory cortex (AP +3.20 mm from bregma: ML

84

+2.06 mm from bregma). These two regions were chosen as frontal is optimum for detection of slow-waves during NREM, whereas central regions are optimum for detection of sleep spindles (Oishi et al., 2016). The ground was placed in the right medial frontal lobe (Approximately: AP +2.49 mm, ML -1.15 mm from bregma). The common reference was placed below lambda (AP -6.75 mm, ML -0 mm from bregma). Two Teflon-insulated stainless-steel wires (Cooner Wire) with the distal three millimetres exposed were inserted bilaterally into the dorsal nuchal neck muscle posterior to the skull to provide EMG data. Dental cement was used to cover the exposed wires. However, before dental cement was applied, a viscous eye gel was applied to prevent any leakage of cement contacting the eye (Viscotears, Alcon, UK). All mice were allowed to recover for seven days following the procedure before any testing took place.
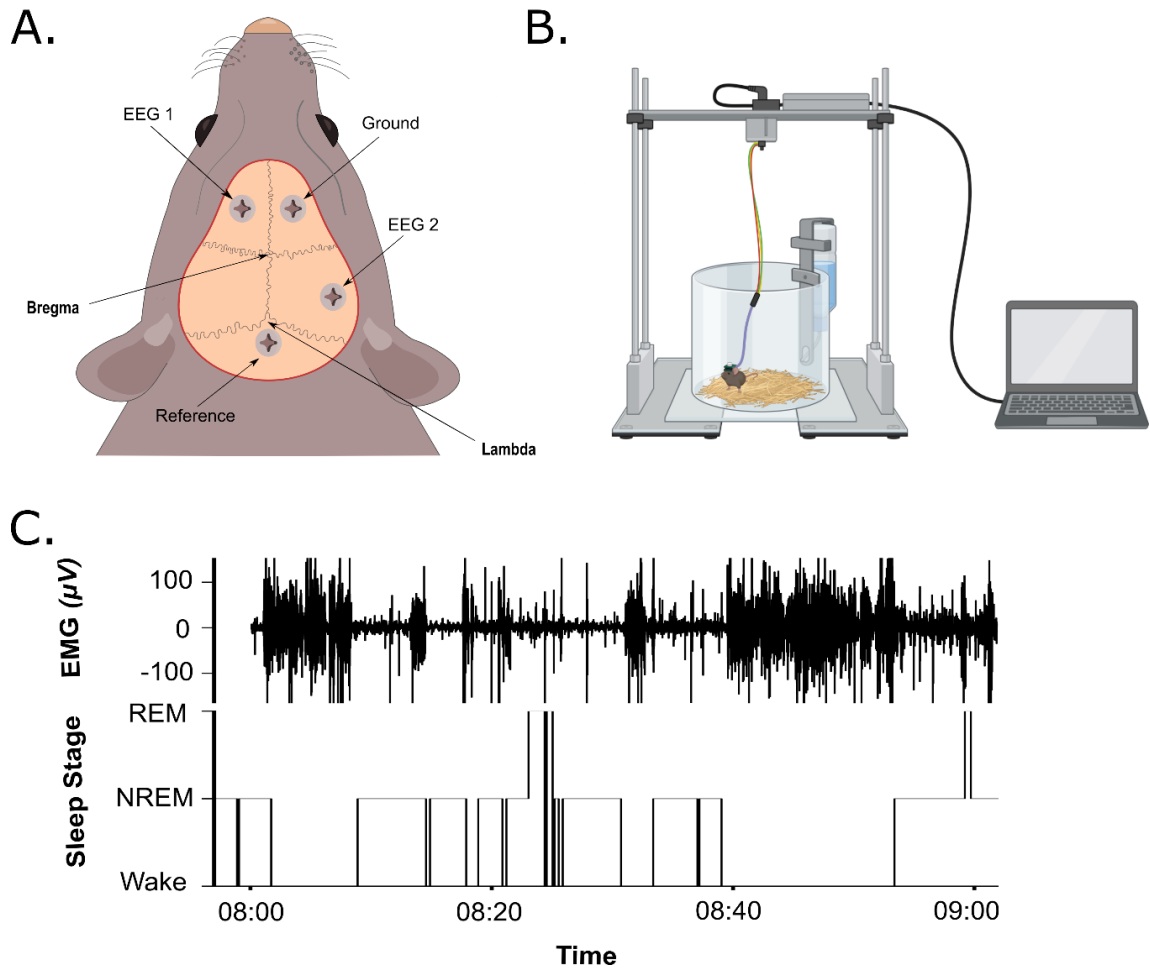
Figure 21: Schematic of the EEG surgery and recording setup
A) The locations of the placement of stainless-steel screw electrodes for EEG recording. **B)** the recording set up of the Pinnacle Technology Inc sleep system. **C)** A sample EMG trace from one representative mouse during the baseline recording period, and the corresponding hypnogram demonstrating the sleep stages and transitions. Abbreviations: EMG = electromyography, REM = rapid-eye-movement sleep, NREM = non-rapid-eye-movement sleep.

EEG Acquisition

Mice were single housed and had access to normal food, water, bedding, and nesting material for the duration of recording. EEG acquisition was conducted using Sirenia® acquisition software (Pinnacle Technology, Inc.), and video data using a USB infrared camera. EEG data was acquired with a sampling rate of 1000 Hz and band-pass filtered from 0.5-20 Hz EMG was band-pass filtered from 10-100 Hz.

The EEG data were manually sleep scored in 10 second epochs using Sirenia®
Sleep Pro software (Pinnacle Technology, Inc.). Each epoch was scored as either REM
sleep, NREM sleep, or wake in line with conventional guidelines. The time in minutes
spent in each sleep stage were recorded and plotted on hypnograms for visualisation.
For statistical analysis, the minutes spent in each sleep stage (NREM, REM, wake),
mean sleep stage length (in minutes), total sleep stage shifts, arousals (>10s wake after
NREM/REM sleep), NREM and REM sleep latency (in minutes), and the number of
sleep stage episodes and mean sleep stage episode duration was computed for each
mouse. The percentage of total sleep (both NREM and REM) and number of arousals
over the recording period was also computed. REM and NREM were combined to give
an overall measure of sleep and visualised in 1-hour windows. Cumulative probability
distributions of the duration of stage bouts (in minutes) were computed for each sleep
stage.

A well validated proxy measure of sleep deprivation is to measure the sleep
rebound – whereby after sleep deprivation there is an increase in the depth of sleep in
subsequent recovery sleep (Rechtschaffen et al., 1999). This is typically measured by
computing the PSD in the delta band (1-4 Hz) during NREM sleep (Brunner et al.,
1990). To establish this, a Welch's Fast Fourier Transform (FFT) was computed on the
time-series data to produce absolute PSD (μV^2/Hz). Welch's method was chosen over
other FFT methods as this averages the periodograms over short segments of the
windows and reduces the variance (Verma & Dekar, 2018). PSD from both EEG
channels (EEG1 – frontal, EEG2 – central) was calculated for 0.5 frequency bins (0.5
– 20 Hz). Relative delta (1-4H Hz) PSD was calculated as the power within the delta

band (μV^2/Hz) divided by the total power (μV^2/Hz) across all frequencies (0.5 – 20 Hz).

2.4 Sleep Deprivation

To achieve total sleep deprivation, the gentle handling method (GHM) (Franken et al., 1991; Mang & Franken, 2012) was used. Previous studies have found a complete abolition of REM sleep and reduction of NREM sleep to 92% and 98% of baseline levels respectively (Alexandre et al., 2017; Franken et al., 1991). Mice were left in their home cages with nesting material removed. Mice were left relatively undisturbed until they showed signs of sleep (>5 seconds of immobility). If the mouse attempted to sleep, a novel object (plastic toys, Lego bricks etc.) was gently introduced into the cage. If the mouse attempted to sleep after this, then new nesting material was provided to the cage, and the cycle was repeated for the duration of the sleep deprivation period. Mice were sleep deprived from ZT0–ZT6, corresponding to lights on (07:00) to 13:00. Control mice were left to sleep in their home cages in the same room.

2.5 Assessment of Circadian Rhythms

For analysis of circadian activity patterns, mice were single housed and placed in a circadian cabinet (Phenome Technologies, Inc.) for 1 week to acclimatise to the environment under a normal 12-hour light-dark cycle (lights on 07:00 – 19:00). The cabinet was in a light controlled, sound-proofed room which was only used to house the cabinets. Light, humidity and temperature were controlled using ClockLab Chamber Control software (ActiMetrics, USA). The software can control the intensity and timing of light-emitting diode lights to enable manipulation of light/dark cycles. The cabinet measures activity counts using passive infrared sensors (PIR) above each individual cage. Mice were randomised across the cabinet in terms of male and female and always placed in the same location across the course of an experiment. Cages were

not changed during the experiment as this has been shown to induce a significant circadian phase shift (Unpublished Observation). Mice had access to food and water ad libitum, and normal nesting material whilst housed in the cabinets. When mice were placed under constant dark conditions, red light was used when entering and exiting the room and the mice had no exposure to other light cues (Brown et al., 2020). Data were acquired using ClockLab 4 (ActiMetrics, USA) software on a personal computer. Clocklab Analysis 6 (Actimetrics, USA) was used to analyse activity counts, activity profile, activity onsets relative to lights off, bouts, sleep (%), and period length as detailed in results chapters. Period length (τ) was determined by chi-squared (χ^2) periodogram analysis at 1-minute resolution. The χ^2 periodogram is a common application of the Enright periodogram, which is widely used for circadian period estimation (Enright, 1965). Sleep percentage was calculated from the number of sleep epochs divided by the total number of epochs multiplied by 100. A proxy measure of sleep epochs was calculated whereby if activity count = 0 for an epoch (epochs = 1 minute) the mouse was assumed to be asleep. To determine the duration of the activity period (α), the daily onset and offset times of locomotor activity were identified, and the intervening time was calculated. For each animal, α was averaged over a 7-day period in light/dark conditions.

2.6 Behavioural Testing

2.6.1 Mechanical Threshold Assessment (von Frey Assay)

<u>von Frey Habituation</u>

In order to assess cutaneous orofacial sensitivity in mice the established von Frey assay was used (Chaplan et al., 1994). The behavioural testing room was maintained between 30-50 lux and ~21°C and was separate to the vivarium. All testing was

conducted at the same time each day to avoid any circadian variation (Minett et al., 2014). Mice were habituated to the von Frey apparatus for three days before baseline measurements by placing them in the apparatus (a clear Perspex box) in a 4 oz paper cup for 1 hour. After this, the experimenter removed each mouse from the box and placed them in the cup until they sat upright with their face out of the cup and placed their forepaws on the rim. Mice were then returned to their home cages. Each mouse was always placed in the same cup and the same box each time the test was conducted. Initially, mice were always placed in a blinded and randomised fashion (according to genotype or experimental group).

von Frey Periorbital Threshold Testing

Mice were habituated in the same manner as described above for 1-hour prior to each sensory testing instance. The mouse and corresponding cup were removed from the box, and once the mouse was sat upright, the first von Frey filament (0.4 g) was applied to the periorbital region. If the mouse withdrew from the filament into the cup or brushed the area with their forepaw this was marked as a positive response. If the mouse did not respond this was marked as a negative response. Filaments were tested using the up-down method (Chaplan et al., 1994). Baseline thresholds were measured in this manner three times with 3-4 days in between, to give three baseline measurements. As mice can anticipate the filaments when they are unfamiliar thus leading to potential false positives, the first baseline was discarded and then the last two baselines were then averaged to give an overall baseline measure. The entire testing took no longer than 15 minutes. Each mouse was placed in their home cage and returned to the housing room following final sensory testing. On experimental days this process was repeated.

Mechanical withdrawal thresholds were then calculated using the following formula:

$$50\% \, threshold(g) = 10(X + kd)/104$$

Where X = the value (in log units) of the final von Frey filament, k = tabular value for the response and d = the average increment (in log units) between the filaments (see **Appendix 1**). Based on their basal thresholds, mice were counterbalanced into respective experimental groups and any differences between groups would be statistically assessed to ensure a similar average basal threshold across them.

von Frey Hind Paw Threshold Testing

The testing was conducted as above; however, the mice were placed directly into the Perspex boxes. The von Frey filaments were instead applied to the plantar surface of the hind paw (see **Figure 22**). The filament was applied to the same paw for each mouse and across each experimental group. A positive response was recorded if the mouse licked the paw or moved the paw away from the filament immediately after it was applied. A slight flex in the paw but no movement was counted as a negative response. The 50% mechanical withdrawal thresholds were calculated as above.

Figure 22: The von Frey method for measuring mechanical allodynia
A) Location of where the von Frey filaments are applied to the periorbital region B) Location of where the von Frey filaments are applied to the hind paw.

2.6.2 Photophobia

As a readout of photophobia, a plexiglass chamber with two compartments (20 cm width x 20 cm length each) was used. One compartment was dark and covered. The other chamber was a brightly lit box at 6000 lux, with a USB camera above to record the mouse's movements. There was a small door between the compartments for the mouse to transition between chambers. The mouse was placed in the light chamber facing away from the entrance to the dark, and video recorded for 10 minutes. The resulting videos were scored blind to condition and session. The resulting measures were time spent in the light chamber in seconds, the number of transitions between the light and dark compartment, the latency to first enter the dark compartment in seconds, and latency to re-enter the light compartment after first entry to the dark compartment in seconds.

2.6.3 Open Field Test

Locomotor activity was measured using an infrared photobeam apparatus (MotorMonitor, Kinder Scientific). The apparatus was in a dim room 30-50 lux. Mice were placed individually in a square plexiglass arena (72 cm length x 72 cm width x 33 cm height) which records movement by assessing how often beam breaks occur and allows measurement of basic (gross) and fine movements and entry and time spent in defined zones. The zones in this experiment were defined as follows: Zone 2 = center of the box (measuring 36 x 36 cm), Zone 1 = outer area of the box. A mouse was placed in each box and randomized according to experimental condition and sex. Boxes were cleaned thoroughly between mice and no experimenter was present in the room during recording. Mice were habituated to the apparatus for eight minutes 24 hours before initial testing. As locomotor activity can show diurnal variation, mice were always tested at the same circadian time. Beam break counts were reduced by the software and analysed per 10 second intervals. Counts were automatically divided by the analysis software into gross movements (large movements around the enclosure across multiple area) and fine movements (small movements within one area). As this test is also used to measure anxiety-like behaviours, when used to assess locomotor activity mice were habituated to the arena twice before testing.

2.6.4 Thermal Pain Thresholds

The mouse was placed on a temperature-controlled plate (Ugo Basile, Hot Plate 35100-002) which was heated and maintained at 52°C within a Perspex cylinder. The time was recorded as soon as the mouse's hind-paw contacted the plate. A response was recorded if the mouse licked their hind-paw or reared with a flick of the fore or hind paw. The mouse was taken off the plate immediately after they responded and the maximum placement on the plate was 20 seconds to prevent any damage to the paws.

All testing across experimental groups or genotypes was conducted at the same circadian time as diurnal variation has been reported in hotplate latencies (Crockett et al., 1977). Moreover, previous work has shown reduced latencies with repeated testing sessions (Crockett et al., 1977), as such, we conducted only two testing sessions and the average of this was taken as the hotplate withdrawal latency.

2.7 Biochemistry and Histological Analysis

2.7.1 Tail Vein Blood Sampling

The mouse's tail was disinfected with 70% ethanol and a small cut was made using a sterile safety blade. Approximately 50 µl of blood was collected in a Potassium-EDTA CB (300 µl) (Microvette®) tube and held on ice. Pressure was placed on the wound with a sterile gauze and the mouse was placed into a clean empty cage on top of sterile tissue until there was little or no blood coming from the wound. After this, the mouse was returned to its home cage. To minimise the effects of the smell of blood on other mice, one hour after blood sampling mice were returned to the housing room. All blood samples were centrifuged at 12,000 rpm at 4°C for 10 minutes to separate out the plasma. Plasma was then transferred to 50 µl Eppendorf tubes and stored at -20°C.

2.7.2 Perfusion Fixation and Tissue Preparation

Mice were euthanised with 20 mg/ml of pentobarbital (Euthatal) then intracardially perfused with 20 ml of 1% heparinised PBS, followed by 20 ml of 4% paraformaldehyde (PFA) in PBS. The brains, spinal cord, and trigeminal ganglia were removed where needed and post-fixed in 4% PFA overnight at 4°C before transferring to 30% sucrose for 24 hours at 4°C. Brains and spinal cords were then flash frozen in cryoprotective optimal cutting temperature compound on dry ice. Sections were cut

serially on a cryostat at 30 μm, and immediately placed in antifreeze solution (20% glycerol, 30% ethylene glycol in 0.01M PBS) and stored at -20°C until later use.

2.7.3 Immunohistochemistry (IHC)

<u>c-Fos</u>

Neuronal activation in nuclei of interest was determined by c-Fos immunoreactivity. c-Fos is a proto-oncogene and thought to be an indirect marker of neuronal activity as c-Fos is expressed rapidly (within 15 minutes) when neurons fire action potentials and in response to stimuli (Dragunow & Faull, 1989). It is encoded by the immediate early gene Fos (Sheng & Greenberg, 1990). The nuclear protein product c-Fos is commonly visualised using immunohistochemical (IHC) approaches (Hunt et al., 1987). Previous studies have shown c-Fos expression in the brain and spinal cord in response to chemical, thermal and mechanical stimulation (Harris, 1998).

To determine the number of c-Fos positive cells (c-Fos+) in nuclei of interest we used a standard free-floating immunohistochemical (IHC) approach. For each mouse, four coronal sections from the region of interest (ROI) were taken as determined by a mouse brain atlas (Allen Brain Atlas). Sections were washed in phosphate buffered saline (PBS) and 3% hydrogen peroxide (in diH$_2$O). An anti-c-Fos primary antibody (c-Fos 9F6, rabbit mAb – Cell Signalling Technology®, diluted 1:6000) was applied, and following 24 hours incubation at 4°C the secondary antibody (Biotinylated anti-rabbit raised in goat IgG (H+L) (BA-1000, Vector Laboratories), diluted 1:500), was applied. The signal was amplified using an Avidin/Biotin complex (Vectastain ABC Kit, Vector Laboratories). Negative control sections were used which omitted the primary antibody from the diluent to confirm specificity of the primary antibody. Tissue

sections were blocked with 5% normal goat serum throughout. Finally, the sections were incubated with 3,3′-diaminobenzidine (DAB) containing nickel (SK-4100, Vector Laboratories, Burlingame, CA, USA) for 1-5 minutes before being washed in PBS. Sections were mounted onto 1mm Clarity slides (Camlab), air-dried, dehydrated in gradual increasing concentration of ethanol (50%, 70%, 95%, 100%), followed by xylene and mounted using distyrene plasticizer xylene (DPX) (Sigma-Aldrich, UK) mounting medium under a glass coverslip (Menzel™ Gläser).

After drying for 24 hours, sections were visualised under the light microscope (Zeiss AxioPlan) at 10x magnification. Exposure settings were kept consistent across all images, and firstly determined by averaging the auto-exposure values from three random set of images from each experimental group. Brain regions were identified cytoarchitecturally using a mouse atlas (Allen Brain Atlas) and c-Fos+ cells were identified if they had a solid brown-black colour, distinguishable from the background. Images were exported using Zen Pro software (Zeiss). All sections were imaged on the same day to avoid batch effects.

All cell counting was conducted blind to the experimental condition. To minimise experimenter bias, counting of c-Fos+ cells was conducted using a semi-automated macro developed in ImageJ (Rueden et al., 2017), whereby each image was converted to 8-bit and a threshold was set at a consistent value. This threshold was determined based on the average threshold of all the images. The images were converted to a binary mask, watershed to split any overlapping cells, and then the number of cells greater than 200 pixels in area (or the smallest c-Fos+ cell in that image) were counted.

<u>CGRP</u>

To determine the area of CGRP immuno-positive fibres in nuclei of interest we used a standard free-floating fluorescent IHC approach. For each mouse, three coronal sections from each ROI (TNC, C1, C2) were taken as determined by a mouse brain and spinal cord atlas (Allen Spinal Cord Atlas, 2008). Sections were washed in wash buffer (0.1% Triton X-100 in 0.01M PBS), followed by 3% hydrogen peroxide (in diH_2O) for 15 minutes. They were incubated in a blocking solution containing 10% Normal Donkey Serum (NDS; Merck Life Science UK Ltd) for one hour. An anti-CGRP primary antibody (Sheep Anti-CGRP antibody, Abcam, diluted 1:2000 in 0.25% Triton X-100 in 0.01M PBS, with 5% NDS) was then applied, following three five minute washes in wash buffer and the sections were left to incubate for 24 hours at 4°C. Negative control sections were used which omitted the primary antibody from the diluent to confirm specificity of the primary antibody. Following more washes (3x15 minutes) in wash buffer, the secondary antibody (Alexa Fluor® 568 donkey anti-sheep IgG, Invitrogen, UK, diluted 1:1000 in 0.25% Triton X-100 in 0.01M PBS, with 5% NDS), was applied, followed by 4′,6-diamidino-2-phenylindole (DAPI, diluted 1:40000 in diH_2O, Thermo Fisher Scientific) to visualise nuclei. Sections were then transferred to 1mm glass slides (Clarity, Camlab), air-dried, and mounted using ProLong™ Gold Antifade Mountant medium (Thermo Fisher Scientific) under a glass coverslip (Menzel™ Gläser).

After drying for 24 hours protected from light, sections were then visualised using a wide-field fluorescent microscope (Zeiss AxioImager 2) at 10x magnification. ROIs were confirmed cytoarchitecturally using a mouse atlas. Exposure settings were kept consistent across all images, and firstly determined by averaging the auto-exposure values from three random set of images from each experimental group. All sections were imaged on the same day to avoid batch effects.

Determination of CGRP expression was conducted blind to the experimental condition. Expression of CGRP in fibres were counted in the superficial lamina I/II as this is where the majority of first order nociceptive trigeminal nerve endings terminate (Contreras et al., 1982). To minimise any experimenter bias, calculation of CGRP positive fibres was conducted using a semi-automated macro developed in ImageJ (Rueden et al., 2017). Firstly, each image was converted to an 8-bit grayscale image. All images were then used to calculate an average threshold for the intensity of fluorescent staining, which would be set for all subsequent quantification. Images were converted to a binary mask and the percentage of the area in the image which met or succeeded the fluorescent threshold was calculated. Six sections from each mouse were used for quantification (two sections per level: TNC, C1, C2). All resulting frames were averaged and reported as the mean ± standard error of the mean (SEM).

2.7.4 Enzyme-linked Immunosorbent Assay (ELISA)

Corticosterone levels are widely used to provide information on whether there is a dynamic response of the hypothalamic-pituitary-adrenal (HPA) axis in response to a behavioural stressor (De Kloet et al., 1998). Previous research has shown that corticosterone levels rise to a maximum approximately 40 minutes following an acute stressful event (Gong et al., 2015).

Levels of plasma corticosterone were measured using an established Enzyme-linked Immunosorbent Assay (ELISA) kit from Enzo Life Sciences (Farmingdale, NY, kit no. ADI-900-097) according to the manufacturer's instructions. Samples were processed on a 96-well microplate (Corning™ Costar™), diluted 1:25 with assay buffer after initial optimisation and ran in duplicate. Optical density was determined using an absorbance microplate reader (SpectroSTAR® Nano - BMG LABTECH). The detection limit of the assay was 27 pg/ml. Concentrations for each sample were

determined as the percentage bound using a standard curve with detection range between 20,000 and 31.25 pg/ml using a four-parameter logistic curve. The final concentration of plasma corticosterone was reported as nanograms per millilitre.

2.7.5 qPCR – Prepro-orexin

Mice were terminated using cervical dislocation and hypothalamic-enriched tissue in which the cortex was removed, and the inferior part of sagittal sections were taken was snap frozen in liquid nitrogen and stored at -80°C until use. All tissue collection was completed within one hour to avoid any circadian variation across samples.

RNA Isolation and cDNA Synthesis

Tissue samples were homogenized using guanidinium isothiocyanate and RNA was extracted using silica gel-based spin columns (RNEasy® Lipid Tissue Mini Kit, Qiagen, Germany) after digestion of gDNA with deoxyribonuclease I. Complementary DNA (cDNA) was synthesized from 1 µg RNA using the SuperScript™ Master Mix with ezDNase enzyme (Thermo Fisher Scientific) according to the manufacturer's instructions. A negative control without the reverse transcriptase enzyme was included. The absence of amplified DNA fragments in this sample indicated the isolation of RNA free of gDNA.

qPCR

For the qPCR assay, mouse prepro-orexin specific sense and anti-sense oligonucleotide primers were designed using Ensembl (Cunningham et al., 2022) and Primer 3 (Untergasser et al., 2012) as shown in **Table 5.** We used β-Actin as the housekeeping gene. The reaction contained: 5 µl of Express SYBR GreenER SuperMix with Premixed ROX (Invitrogen, A1031), 0.6 µl primer (5 µM per primer) and 4 µl of

RNAse-free H$_2$O for a total reaction volume of 10 µl per well. The plate was sealed and centrifuged in a plate centrifuge (Multifuge™, Thermo Fischer) for 2 minutes, at 4°C at 5,000 rpm. The plate was processed in a thermocycler (Roche LightCycler® 408), following the same program shown in **Table 4**. Absolute quantification (2nd derivative) was selected as the analysis method.

We used the 2-ΔΔCt method (Livak & Schmittgen, 2001) to quantify the relative expression of prepro-orexin to β-Actin. The mean prepro-orexin expression of the WT RNA samples ΔCt was selected as the baseline ($M = 6.84$). ΔΔCt was calculated for all samples and reported as log fold-change expression relative to β-Actin.

Table 5: Primers used in the prepro-orexin qPCR

Primer Name	Primer Sequence	Fragment Size (bp)
β-Actin		
β-Actin Forward	5' - GGC TGT ATT CCC CTC CAT CG – 3'	241
β-Actin Reverse	5' - CCA GTT GGT AAC AAT GCC ATG T - 3'	
Prepro-orexin		
Prepro-orexin Forward	5' - CAG CCT CTG CCC GAC TGC TGT - 3'	270
Prepro-orexin Reverse	5' - TAA AGC GGT GGT AGT TAC GGT CGG AC - 3'	

Abbreviations: bp = base pairs, qPCR = quantitative polymerase chain reaction.

2.7.6 Metabolic Assays

The following protocol was taken and adapted from Professor Alexandra Sinclair's laboratory at the University of Birmingham (UoB). These experiments were conducted in collaboration with Olivia Grech and Professor Sinclair at UoB.

Sample Preparation

Whole brains were snap frozen in liquid nitrogen and stored in cryotubes (Starsedt, Germany) at -80°C until required for analysis. To reduce biological degradation, the sample preparation method was optimised to provide accurate tissue weights whilst avoiding sample thawing. Therefore, samples were placed on dry ice and a polystyrene box was filled with liquid nitrogen and all equipment (pestle and mortar, sample tubes, spatula, tweezers, microspoon and metal funnel) were cooled until temperature equilibration. Preparation of the slices involved pulverisation of the tissue in liquid nitrogen using the mortar & pestle (ThermoFisher Scientific) lined with tinfoil. Powdered tissue was then poured back into the cold cryotube using the tinfoil and a cooled metal funnel. Typically, 30 mg of pulverised tissue was then weighed out for nuclear magnetic resonance spectroscopy (NMR) analysis. Powdered sample was weighed in pre-cooled sample tubes in a calibrated microbalance (Accuris Instruments Analytical Series W3100-210, Dublin, Ireland) in an upright holder.

Metabolite Extraction

500 µl HPLC grade methanol at -20°C was added to tissue in sample tubes to quench metabolism followed by 200 µl of 2.5 µg/ml glutaric acid in ddH$_2$O as an internal standard. A 5 mm steel bead (QIAGEN) was added, and samples were placed into a cassette cooled to -20°C and homogenised using a TissueLyser II (QIAGEN) at 30 cycles per second for two minutes, then centrifuged at 15,000 rpm for 10 minutes.

The supernatant was extracted and moved to a glass tube in which protein contaminants were precipitated by adding 2:1 acetone:isopropanol solution at -20°C. Samples were vortexed then agitated for 10 minutes on an orbital shaker at 750 rpm (Fisherbrand multi-tube vortexer) and centrifuged at 3220 x g for five minutes to form a protein pellet. The supernatant was transferred to a fresh glass tube and the protein pellet was left to dry overnight, the weights of which were used for normalisation. H_2O (HPLC grade) with chloroform was added to the supernatant at -20°C to aid separation of non-polar metabolites. Samples were agitated as before then centrifuged at 4000 rpm for 5 minutes. 1.8 ml of the polar fraction was transferred to a new tube and evaporated to dryness using a SpeedVac (ThermoFisher Scientific) at 30°C for 4-5 hours and stored at -80°C until further analysis. 200 µl of the polar fraction was transferred to another tube for GCMS analysis, dried using the SpeedVac (ThermoFisher Scientific) at 30°C for 4-5 hours and stored at -80°C until further analysis.

Pellet Resuspension

Sample pellets were resuspended in 60µl of 100 mM sodium phosphate buffer made in 1L dH_2O (mM): Na_2HPO_4 (57.8), NaH_2PO_4 (42.2), D4-TMSP (0.5 mM) pH 7.0 then briefly vortexed and then sonicated for five minutes. 50 µl was pipetted into champagne vials and a Gilson liquid handling robotic system (Cortecnet) transferred 35 µl into 1.7 mm NMR tubes.

NMR Data Acquisition

NMR was utilised for quantification of metabolites. NMR tubes containing samples were loaded into Bruker Neo 800 MHz NMR spectrometer, equipped with a 1.7 mm z-PFG TCI Cryoprobe. A NOESY1d pulse sequence was used to achieve water

suppression. Automatic tuning and matching were used and samples were shimmed to a Trimethylsilylpropanoic (TMSP) acid line-width of <1Hz. In total 128 transients with 16384 complex data points (1.31 seconds FID acquisition time) were recorded for each sample, with a total of 16 steady-state scans per sample. The interscan relaxation delay was set to 4 seconds and the spectral width of 12500Hz (15.63 ppm). Total acquisition time for each spectra was ~13 minutes. Data acquired was in the form of 1H 1D-NMR spectra.

<u>NMR Data Analysis</u>

1H 1D-NMR spectra were analysed using MetaboLabPy (version 0.6.35, Ludwig 2020). The D4-TMSP signal was set at 0ppm and chemical shifts were calibrated accordingly. Spectra were manually phase corrected before baseline correction, and peaks were spatially aligned. The area under each peak of interest was integrated and compared to the integrated area under the TMSP peak (internal reference) to calculate the μM concentration. Dried protein pellet weight was used for normalisation.

2.8 Pharmacological Agents

Vehicle control animals were always treated with saline (0.9% NaCl).

2.8.1 Nitroglycerin

Nitroglycerin (NTG; Hospira-Pfizer, UK) stock solution (25 mg/kg) was diluted fresh in saline (0.9% NaCl) to a dose of 10 mg/kg prior to intraperitoneal (i.p.) administration (1:5). As it binds to soft plastics, NTG was prepared in glass vials. The resulting solution was protected from light and used immediately. All injections were given at a volume of 10 ml/kg.

2.8.2 Orexin-A (OXA)

500 µg of stock solution of OXA (Tocris BioScience) was dissolved in 2000 µl of saline. OXA was given i.p at doses of 100 µg/kg and 40 µg/kg based on the literature (Becquet et al., 2019; Öz et al., 2018). OXA was dissolved in 0.9% saline prior to administration. All injections were given at a volume of 10 ml/kg.

2.8.3 Caffeine

Caffeine dissolved in saline (Caymann Chemicals) was given i.p. at a dose of 20 mg/kg based on the literature (Alexandre et al., 2017).

2.9 Statistical Analysis

Unless otherwise stated all statistical analysis and data visualisation was conducted in RStudio (version: 2022.12.0+353).

Chapter 3: Sleep Quality and Architecture in Migraine

The work in this chapter is published as a peer-reviewed publication: Stanyer, E. C., Creeney, H., Nesbitt, A. D., Holland, P. R., & Hoffmann, J. (2021). Subjective sleep quality and sleep architecture in patients with migraine: a meta-analysis. *Neurology*, *97*(16), e1620-e1631.

3.1 Introduction

As highlighted in **Chapter 1,** the relationship between sleep and migraine is complex, as alterations in sleep can be a trigger, treatment, or symptom of migraine (Kelman, 2007). Whilst there is evidence from both preclinical and prospective clinical research (Bertisch et al., 2019; Kilic et al., 2018; Kim et al., 2019) that links sleep with migraine there remains a poor understanding of sleep in migraine. A clearer understanding of the profile of sleep in migraine and its relation to migraine-related disability is important, to enable clinicians to support those with migraine and deliver targeted, effective sleep interventions (Bellesi et al., 2014).

For example, there remains a paucity of research into sleep in patients with migraine, and there is no consensus on whether they exhibit objective changes in sleep architecture. This is partly due to the small sample sizes of polysomnography (PSG) studies used to measure sleep. In this chapter, we conducted a meta-analysis which aims to overcome this by aggregating data from multiple studies investigating differences in subjective sleep quality and objective sleep architecture between patients with migraine and healthy controls.

Various methods exist to establish subjective sleep quality in healthy individuals (Fabbri et al., 2021). However, the most widely used is the Pittsburgh Sleep Quality Index (PSQI) (Buysse et al., 1989). The PSQI contains nine questions centred on individual's sleep habits during the past month and has been shown to have high test-retest reliability and validity (Backhaus et al., 2002a). The PSQI covers seven areas related to sleep including: subjective sleep quality, sleep latency, sleep duration, habitual sleep efficiency, sleep disturbances, use of sleeping medications, and daytime dysfunction over the last month (see **Table 6** for an overview). However, notably this is self-reported data and thus prone to erroneous recall and bias. PSG on the other

hand relies on the measurement of brain activity using EEG, muscle movement using electromyography, and eye movement using electrooculography (Rundo & Downey, 2019). Other measures supplement this including heart rate, oxygen saturation, and respiration and thus PSG is a more objective measure of sleep. PSG and subjective measures of sleep may not be well related across all measures thus investigating the two together could provide a more holistic picture of sleep in migraine (Cudney et al., 2022; Westerlund et al., 2016).

Additionally, few studies have investigated how poor sleep quality relates to the extent of the impact of migraine on daily life – migraine-related disability. This is important as migraine is a severely disabling disorder (Vos et al., 2017), thus understanding how sleep quality might be related to disability would further our understanding of this relationship. Migraine disability can be measured using the Migraine Disability Assessment Test (MIDAS) (Stewart et al., 2001), which has been shown to have high reliability and validity (Stewart et al., 2000). In the current meta-analysis, we pooled correlational data from existing studies on mean PSQI and MIDAS scores in people with migraine.

Table 6: Main components of the Pittsburgh Sleep Quality Index

During the past month, how often have you had trouble sleeping because you:	Not during the past month (0)	Less than once a week (1)	Once or twice a week (2)	Three or more times a week (3)
A. Cannot get to sleep within 30 minutes				
B. Wake up in the middle of the night or early morning				
C. Have to get up to use the bathroom				
D. Cannot breathe comfortably				
E. Cough or snore loudly				
F. Feel too cold				
G. Feel too hot				
H. Have bad dreams				
I. Have pain				
J. Other reason (s), please describe, including how often you have had trouble sleeping because of this:				
During the past month, how often have you taken medicine to help you sleep?				
During the past month, how often have you had trouble staying awake while driving, eating meals, or engaging in social activity?				
During the past month, how much of a problem has it been for you to keep up enthusiasm to get things done?				

During the past month, how would you rate your sleep quality overall?	Very good (0)	Fairly good (1)	Fairly bad (2)	Very bad (3)

Questionnaire adapted from Buysse et al. (1989).

3.1.1 Review Questions:

1) Is there a difference in subjective sleep quality as measured by the PSQI between people with migraine and healthy controls? Is the effect size larger in those with chronic migraine versus episodic migraine?

2) Are there differences in sleep architecture as measured by PSG between people with migraine and healthy controls, in both adult and pediatric patients?

3) Is there a significant positive correlation between subjective sleep quality in adult migraine patients and migraine-related disability as measured by the PSQI and MIDAS questionnaire respectively?

3.2 Methods

The format of this systematic review followed the Preferred Reporting Items for Systematic Reviews and Meta-analysis (PRISMA) guidelines (Page et al., 2021) and the PRISMA 2020 checklists (see **Appendix 2**). The protocol for this meta-analysis was pre-registered with PROSPERO (registration number: CRD42020209325).

3.2.1 Search Strategy

Two authors (Emily Stanyer and Hannah Creeney) conducted an independent, electronic search of relevant databases [Embase (1996 - 2020), MEDLINE® (1996 - 2020), Global Health (1973 – 2020), APA PsycINFO (1806 - 2020), and APA PsycArticles Full Text (2020)] from their inception to the current date. Key search terms included combinations of migraine, sleep*, PSQI, Pittsburgh Sleep Quality Index, polysomnograph*, PSG, EEG, electroencephalograph*, MIDAS, HIT-6, MSQ with Boolean operators. The full search strategy for OVID is displayed in **Table 7**. The search was limited to studies published in English and duplicates were removed. Titles and abstracts were independently screened for eligibility by two authors. Studies that

were eligible or if eligibility was unclear were submitted to the full text review stage. Relevant studies were also retrieved from the reference lists of eligible studies. All full texts were screened for eligibility by the thesis author and 10% of the full texts were selected randomly using a random number generator which a second reviewer then screened for eligibility. There were no discrepancies between the two authors in classifying eligibility. All studies were examined to ensure they were independent of one another. If full texts were not available, the original authors were contacted, and the full texts were requested. The last search date was the 17th of December 2020.

3.2.2 Inclusion Criteria

Studies were eligible if they examined sleep quality as assessed by the PSQI in adults and/or physiological sleep variables using PSG in adult and pediatric patients with migraine and healthy control participants. Studies which computed correlations between MIDAS and PSQI scores in people with migraine were also included.

3.2.3 Exclusion Criteria

Review articles or single-case studies were not included. Studies which did not have a suitable control group were excluded.

Table 7: Search strategy for OVID

Search number	Keyword/s	Number of results
1	migraine*	128610
2	MIDAS	4457
3	Migraine Disability Assessment	2154
4	MS-Q	286
5	HIT-6	1283
6	Headache Impact Test	1441
7	sleep quality	63290
8	PSQI	12845
9	Pittsburgh Sleep Quality Index	22948
10	EEG	244685
11	electroencephalograph*	333289
12	PSG	19344
13	polysomnograph*	82692
14	1 or 2 or 3 or 4 or 5 or 6	131646
15	7 or 8 or 9	63520
16	10 or 11 or 12 or 13	500081
17	15 or 16	552637
18	14 and 17	4934
19	Limit 18 to English language	4155
20	Remove duplicates from 19	4089

3.2.4 Population

There were no restrictions on the age of the participants in the studies. However, for analysis purposes, adults ≥18 years of age and children <18 years of age were included in separate analyses given that sleep architecture demonstrates age-dependent quantitative differences (Vitiello, 2006). Pregnant participants, and participants with other headache disorders namely: cluster headache, tension-type headache, and medication overuse headache were not included in the analysis. However, a study was included if it reported data which could be extracted that was specific to migraine and no other headache disorders. Due to the limited number of studies in this area, we deliberately kept the classification of migraine broad, thus migraine patients with any diagnosis were included in this analysis: episodic, chronic, MoA, MA. Although this is not a standard definition in the International Classification of Headache Disorders-III (The International Classification of Headache Disorders, 3rd Edition, 2018), studies which also categorized their patients as sleep-related migraine (SRM), or non-sleep-related migraine (NSRM) were included. Studies which categorized their patients by the number of migraine or headache days per month, and not by episodic or chronic were also included. In such cases, we categorized patients experiencing headache/migraine on ≥15 days per month as chronic (as per ICHD-III criteria) and pooled them with the other studies in chronic patients. As sleep quality may differ between patients with different frequencies of migraine, episodic and chronic migraine were analyzed as subgroups in the meta-analysis of PSQI scores.

3.2.5 Outcome Measures

To indicate whether people with migraine experience altered sleep, the primary outcomes calculated were weighted effect sizes (Hedges' *g*) for the difference between people with migraine and controls in global PSQI score and PSG-derived sleep

measures. The PSG-derived sleep measures were total sleep time (TST) in minutes, sleep efficiency percent (SE), percent wake, percent of TST spent in REM sleep, NREM sleep stage 1 (N1), stage 2 (N2), and stage 3 (N3), sleep onset latency (SOL) in minutes. Secondly, to ascertain whether subjective sleep quality is related to migraine disability the Fisher's z transformed correlation coefficient (Silver & Dunlap, 1987) between MIDAS and PSQI scores in the migraine population was calculated.

3.2.6 Data Extraction

Data extraction was performed using an a priori elaborated table in Microsoft Excel. Extraction was completed by the thesis author and included: authors, year of publication, journal, publication type, database extracted from, participant demographics (mean age, sex ratio), migraine characterization, mean global PSQI score, PSG-derived sleep variable means, correlation coefficient between MIDAS and PSQI scores, standard deviations (SD) for each data point, and group sizes. Where the measures were not reported directly in the publication or the data were not in the correct format for analysis (e.g., medians instead of means), the authors were contacted to request this data. Studies which did not report data from which the effect sizes could be calculated after contacting the authors, or if the authors did not respond were excluded.

Further data were extracted that might be potential moderators where appropriate. For example: the design of the study (e.g., matched, or non-matched controls), whether the study excluded those on medication which may affect the sleep cycle or those with co-morbid sleep disorders, presence of a PSG adaptation night or not, and whether the study required adherence to ICHD-III (The International Classification of Headache Disorders, 3rd Edition, 2018) criteria to define migraine. If studies used older sleep scoring criteria (Rechtschaffen, 1968) to determine NREM

sleep stages separately (stage 3 and stage 4 sleep), an average of the means for these two stages was computed to be comparable to updated American Academy of Sleep Medicine Manual (Berry, 2015) nomenclature: which defines these singularly as N3. If a study reported PSG sleep stage variables in minutes rather than percentages, these were calculated manually based on the TST means available.

3.2.7 Statistical Analysis

Statistical analysis was performed using the R *metafor* package (Viechtbauer, 2010). The weighted effect sizes (Hedges' *g)* for each study were calculated using the means, sample size (*n*) and SDs for each of the measures. Effect sizes were interpreted as small (0.2), medium (0.5) or large (0.8) in line with conventional guidelines (Hedges et al., 2012) and were visualized using forest plots. The effect sizes were calculated such that a negative Hedges' *g* value indicated that the controls had a higher score on that measure. For the PSQI analysis in adults, studies which investigated chronic cases were initially pooled with episodic cases for a global analysis of effect size, however they were then analyzed in two separate sub-groups. For the sleep architecture analysis, PSG-derived variables from pediatric and adult patients were analyzed as two separate groups. Effect sizes were computed for each PSG measure separately.

3.2.8 Publication Bias

Publication bias refers to the over-inflation of effect sizes due to the tendency for non-significant findings to remain unpublished. To assess this, Egger's regression test for funnel plot asymmetry was performed (Egger et al., 1997). If the result is significant at $p < 0.05$, this provides evidence of publication bias. However, as Egger's test is prone to producing false positives particularly with small numbers of studies, publication bias was also assessed by visual inspection of funnel plots. These plot a measure of

precision (standard error) against the observed effect size (Hedges' g). If the funnel plots are substantially asymmetric, then publication bias can be assumed. Duval and Tweedie's trim-and-fill method (Duval & Tweedie, 2000) was used to assess whether there were any unpublished studies missing from the analysis and estimate what the adjusted effect size would be if these studies were present.

3.2.9 Between-studies Heterogeneity

As a meta-analysis typically includes studies of varying designs and populations, it is important to quantify the proportion of between-studies heterogeneity to ensure accurate estimation of the summary effect sizes. Cochran's Q statistic is commonly used to assess this. If Q is significant at $p < 0.05$ this indicates variability in the effect sizes reported between studies. However, as this test has poor power to detect heterogeneity when the meta-analysis includes a small number of studies the I^2 statistic was also calculated (Higgins & Thompson, 2002). An I^2 value of 0% represents no heterogeneity, 25% low, 50% moderate and 75% high. As heterogeneity was to be expected given the variation in participant characteristics and experimental designs, a random-effects model meta-analysis was conducted for all measures.

To explore potential sources of heterogeneity in both the PSQI and PSG analyses, where a significant effect size and moderate heterogeneity as indexed by an I^2 of >50% were found, additional analyses were conducted by including characteristics of the study as moderator variables. The variables which were included as moderators were not predefined and were based on a previous meta-analysis (Mathias et al., 2018): exclusion of participants with sleep disorders (yes/no), exclusion of participants taking drugs which affect the sleep cycle or a washout period before the study (yes/no), whether the control population was matched for sex and age (yes/no). Where a study

did not state in the paper whether this was conducted or not, it was coded as 'no' for the purposes of analysis.

3.2.10 Study Quality Assessment

An adapted version of the Newcastle-Ottawa scale (Wells et al., 2000) (NOS) was used to assess the quality of the case-control and cross-sectional studies included in the meta-analysis (**Appendix 3**). For case-control studies: adequacy of case selection, representativeness of cases, selection of controls, and definition of controls was examined for which a maximum of one star (*) could be awarded for each. Comparability of the cases and controls was also examined for which a maximum of two stars could be awarded to each study. For cross-sectional studies, sample representativeness, sample size, non-respondent rate, and comparability of cases and controls was examined (**Appendix 4**). Two authors (Emily Stanyer and Hannah Creeney) independently scored each study based on the NOS criteria. Discrepancies in study assessments were then resolved through discussion. In the case of any discrepancies which could not be resolved, these were resolved by a third reviewer who gave the final decision. The study quality scores (0-6, 0 = low quality, 6 = high quality) for each study were then included as a variable in the moderator analyses.

3.3 Results

3.3.1 Description of Studies

Results of the study selection process are displayed in **Figure 23**. From a total of 4089 studies after duplicates were removed, 183 were identified as potentially eligible after screening the titles and abstracts. The full texts were then screened to confirm this, after which 32 independent studies were included in the meta-analysis. **Table 8** presents an overview of the studies included in the review. There were 23 case-control studies and nine cross-sectional studies. Although categorizing patients

based on ICHD criteria was not an eligibility requirement, this was assessed during study quality assessment and all studies used these guidelines to assess their patients. Twenty investigated sleep quality in adults with and without migraine using the PSQI. Of these, 14 of them had a population consisting of episodic and six of them chronic migraine patients. Four studies (two already included in the PSQI analysis) reported the correlation between MIDAS and PSQI scores in people with migraine (Dikmen et al., 2015; Sadeghniiat et al., 2013; Sengul et al., 2015; Yon et al., 2020). There were no participants <18 years of age in the PSQI analysis or the MIDAS and PSQI correlational analysis due to a lack of available data. Eleven studies measured PSG in adults (Engstrøm, Hagen, Bjørk, Gravdahl, et al., 2013; Karthik et al., 2013; Kristiansen et al., 2011; Marca et al., 2006; Nayak et al., 2016; Vollono Catello et al., 2013) and children (Armoni Domany et al., 2019; El-Heneedy et al., 2019; Esposito et al., 2013; Masuko et al., 2014; Roccella et al., 2019), with and without migraine. One study (Engstrøm et al., 2013) compared PSG from migraine patients in the pre-, mid - and post-ictal phase, and thus it was not appropriate to extract a single value for this data. However, they provided a pooled value for the PSQI analysis, and thus it was excluded from the PSG analysis only.
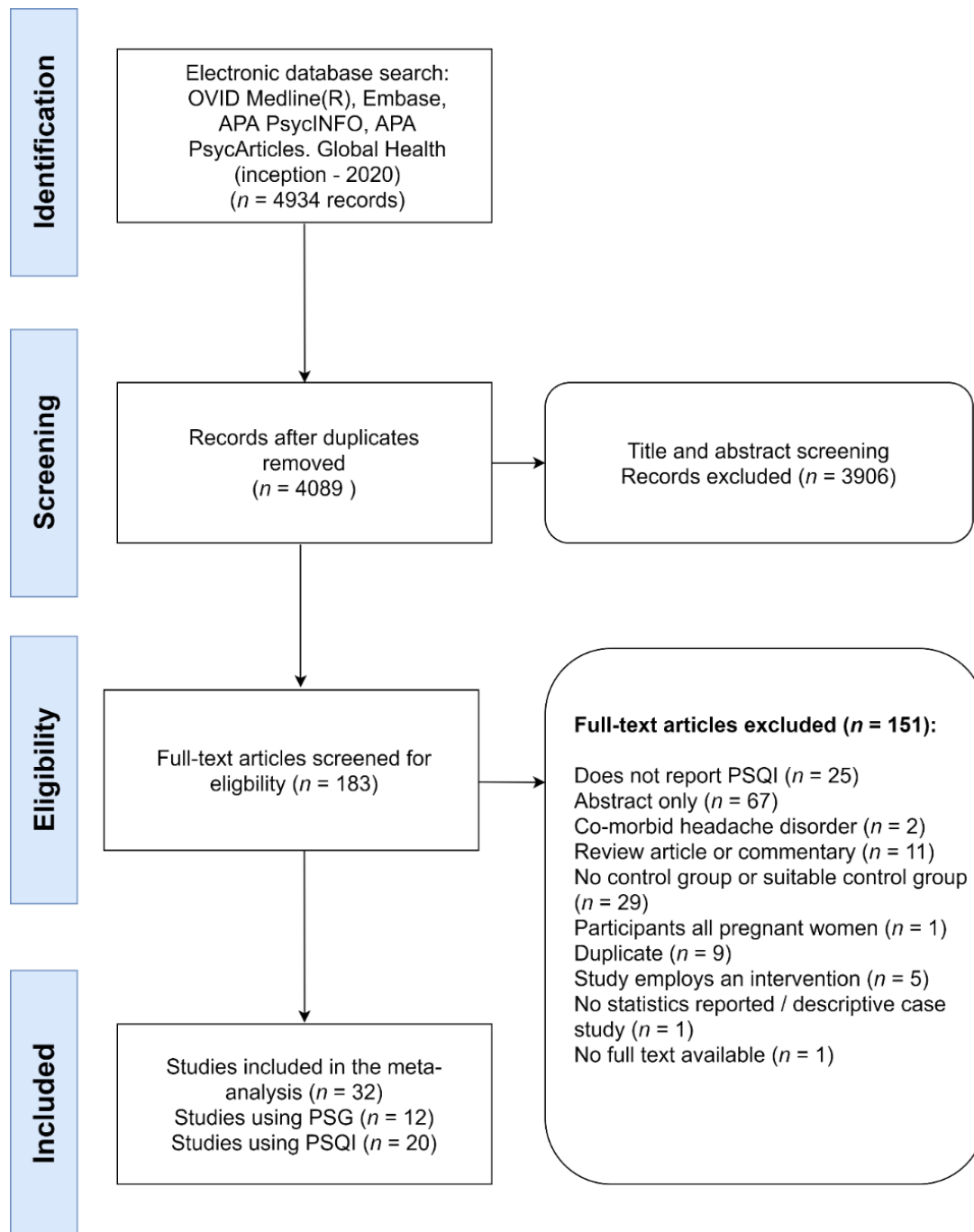
Figure 23: Flow diagram depicting the stages of study selection.
After searching relevant databases and removing duplicates, 4089 abstracts were screened. 183 full-text articles were then screened after exclusion at the abstract stage. This left 32 studies in the meta-analysis. Abbreviations: PSQI, Pittsburgh Sleep Quality Index; PSG, polysomnography; MIDAS, Migraine Disability Assessment.

Table 8: Characteristics of the studies included in the meta-analysis

Reference	*n* total (Mi, C)	Age	Sex ratio (M:F) (Mi, C)	Migraine frequency	Migraine classification	Classified by ICHD criteria?	Control population	Sleep outcomes	Medication exclusion?
Armoni Domany et al. (2019)	365 (185, 180)	13.5 ± 3.4, 9.7 ± 2	72:113, 88:92	episodic	MoA and MA	Yes	Non-matched controls	PSG	No
Barbanti et al. (2007)	200 (100, 100)	36.9 ± 12.1, 36.7 ± 11.7	16:84 (total)	episodic	MoA and MA	Yes	Age and sex matched controls	PSQI	Yes
Barbanti et al. (2013)	200 (100, 100)	46.8 ± 13.8, -	8:92 (total)	chronic	MoA and MA	Yes	Age and sex matched controls	PSQI	Yes
Chu et al. (2018)	244 (65, 179)	32.0 ± 12.6, 34.7 ± 11.9	20:45, 63:116	episodic and chronic	MoA and MA	Yes	Non-matched controls	PSQI	Unknown
Marca et al. (2006)	20 (10, 10)	41.9 ± 13.9, 43.2 ± 16.9	3:7, 3:7	episodic	MoA	Yes	Age and sex matched controls	PSG	Yes – except triptans and NSAIDs
Dikmen et al. (2015)	128 (87, 41)	-	25:62, 16:25	episodic	MoA and MA	Yes	Non-matched controls	PSQI	Yes
El-Heneedy et al. (2019)	60 (40, 20)	11.13 ± 2.85	17:23, -	episodic and chronic	MoA and MA	Yes	Age and sex matched controls	PSG, PSQI	Yes
Engstrøm et al. (2013)	34 (C), 15 (SRM), 18 (NSRM)	33.9 ± 11.4, 39.4 ± 14.3	5:10 (SRM), 3:15 (NSRM), 14:20 (C)	episodic	MoA and MA, SRM and NSRM	Yes	Age and sex matched controls	PSG, PSQI	Yes – except triptans and NSAIDs
Engstrøm et al. (2014)	87 (53, 34)	38.2 ± 12, 39.6 ± 13.7	12:41, 14:20	episodic	MoA and MA	Yes	Age and sex matched controls	PSG, PSQI	Yes – except triptans and NSAIDs
Esposito et al. (2013)	85 (34, 51)	9.08 ± 2.28, 9.37 ± 1.81	20:14, 28:23	episodic	MoA	Yes	Age and sex matched controls	PSG	Yes

Study	N	Age	Sex	Type	Diagnosis		Controls	Measure	
Gori et al. (2005)	200 (78 SRM, 122 NSRM)	40.1 ± 11.2 (total)	22:178 (total)	episodic	MoA and MA	Yes	-	PSQI	Unknown
Karthik et al. (2012)	180 (90, 90)	31.76 ± 8.2, 31.76 ± 8.2	19:71, 19:71	episodic	MoA	Yes	Age and sex matched controls	PSQI	Yes
Karthik et al. (2013)	62 (30, 32)	33.5 ± 8.6 (Mi)	6:24 (Mi)	episodic	MoA	Yes	Age and sex matched controls	PSG, PSQI	Yes
Kozak et al. (2017)	112 (55, 57)	33.31 ± 9,86, 31.58 ± 5.95	8:47, 17:40	episodic	MoA and MA	Yes	Age and sex matched controls	PSQI	Yes
Kristiansen et al. (2011)	533 MOA (71), MA (38), C (431)	-	291:242	-	MoA and MA	Yes	Non-matched controls	PSG	No
Lin et al. (2016)	491 (357, 134)	35.5 ± 12.6 (C)	:89 (C)	episodic and chronic	-	Yes	Age and sex matched controls	PSQI	Unknown
Lin et al. (2016)	505 (372, 133)	32.7 ± 12.4, 35.4 ± 12.6	119:253, 44:89	episodic and chronic	MoA and MA	Yes	Non-matched controls	Chinese PSQI	Unknown
Lin et al. (2019)	273 (96, 177)	32.7 ± 11.9, 35.4 ± 11.3	30:66, 88:89	episodic and chronic	MoA and MA	Yes	-	PSQI	Unknown
Masuko et al. (2014)	40 (20, 20)	9.5 ± 0.48, 9.1 ± 1.7	10:0, 10:0	episodic	MoA and MA	Yes	Age and sex matched controls	PSG	Yes
Morgan et al. (2015)	1060 (145, 915)	35.7 ± 12.1 (total)	423:637 (total)	episodic	MoA and MA	Yes	Non-matched controls	PSQI	Unknown
Nayak et al. (2016)	50 (25, 25)	29.2 ± 5.26, 26.3 ± 7.4	6:19, 6:19	episodic	MoA	Yes	Age and sex matched controls	PSG, PSQI	Yes
Rafique et al. (2020)	206 (103, 103)	21.1 ± 3.3, 22.1 ± 4.1	0:206	episodic	MoA and MA	Yes	Age and sex matched controls	PSQI	No

Study	N (migraine, controls)	Age	F:M	Migraine type	Diagnosis		Controls	Sleep measure	Medication
Roccella et al. (2019)	85 (33, 52)	10.4 ± 2.0, 9.9 ± 2.4	20:13, 29:23	episodic	MoA	Yes	Age, sex and BMI matched controls	PSG	Yes
Rockett et al. (2013)	30 (15, 15)	34.1 ± 10.6, 34.0 ± 10.6	0:30	-	MoA and MA	Yes	Age and BMI matched controls	PSQI	No
Sadeghniiat et al. (2013)	332 total	36.3 ± 10.1	132:200	episodic and chronic	MoA and MA	Yes	No controls	PSQI	No
Sengul et al. (2015)	165 (120, 45)	28.8 ± 8.8, 30.3 ± 7.7	29:91, 11:34	episodic and chronic	MoA and MA	Yes	Non-matched controls	PSQI	Yes – except preventive treatment
Song et al. (2018)	1273 (143, 1130)	-	36:107 (Mi)	episodic	MoA and MA	Yes	Non-matched controls	PSQI	No
Suzuki et al. (2013)	301 (161, 140)	33.1 ± 10.0, 33.1 ± 6.4	35:126, 14:126	episodic	MoA and MA	Yes	Non-matched controls	PSQI	Unknown
van Oosterhout et al. (2018)	2578 (2389, 189)	45.1 ± 11.7, 46.4 ± 14.2	351:2047, 87:102	episodic	MoA and MA	Yes	Non-matched controls	PSQI	Unknown
Vollono Catello et al. (2013)	16 (8, 8)	48.1 ± 9.3, 46.7 ± 10.7	2:6, 2:6	-	SRM	Yes	Age and sex matched controls	PSG	Yes – except triptans and NSAIDs
Walters et al. (2014)	286 (78, 208)	19.2 ± 3.1 (total)	20:58, 68:140	episodic	MoA and MA	Yes	Non-matched controls	PSQI	Unknown
Yon et al. (2020)	42 (21, 21)	33.5 ± 10.1, 32.8 ± 7.23	-	chronic	MoA and MA	Yes	Age and sex matched controls	PSQI	Yes

Abbreviations: "-" = not reported in the paper, C = controls, Mi = migraine. F = female, M = male, PSQI = Pittsburgh Sleep Quality Index, PSG = polysomnography, MIDAS = Migraine Disability Assessment, BMI = Body Mass Index, MoA = migraine without aura, MA = migraine with aura, SRM = sleep-related migraine, NSRM = non-sleep-related migraine, NSAIDs = Nonsteroidal anti-inflammatory drugs; age is reported as mean ± SEM (migraine, controls).

3.3.2 Risk of Publication Bias

There was no indication of publication bias for any of the analyses apart from the PSQI and MIDAS correlation analysis as indicated by Egger's test for funnel plot asymmetry (**Table 9**). The trim-and-fill method estimated that there was one study missing on the right-hand side, and this would increase the overall effect size to significance but not change the direction of effect (z = 0.44, p = 0.024). Although Egger's test was not significant for any of the other analyses, visual inspection of the funnel plots was completed. For the PSG analysis, visual inspection, and the trim-and-fill method (see **Figure 24**) revealed that for wake there was likely to be one study missing on the right. However, adjustment of the effect size did not change the direction of overall effect or reduce the effect size, thus publication bias is unlikely to influence these results.

Table 9: Results of Egger's regression test for publication bias

Analysis	z	*p*-value
PSQI	-0.77	0.441
TST (mins)	0.06	0.953
Wake (%)	-1.28	0.202
SOL (mins)	0.50	0.617
SE (%)	-0.45	0.652
N1 (%)	-1.08	0.280
N2 (%)	1.26	0.207
N3 (%)	0.88	0.378
REM (%)	-0.50	0.614
MIDAS & PSQI	-4.33	<0.001

Abbreviations: REM, rapid-eye-movement sleep; TST, total sleep time; PSQI, Pittsburgh Sleep Quality Index; SOL, Sleep Onset Latency; SE, Sleep Efficiency; N1, non-rapid-eye-movement sleep stage 1; N2, non-rapid-eye-movement sleep stage 2; N3, non-REM sleep stage 3.
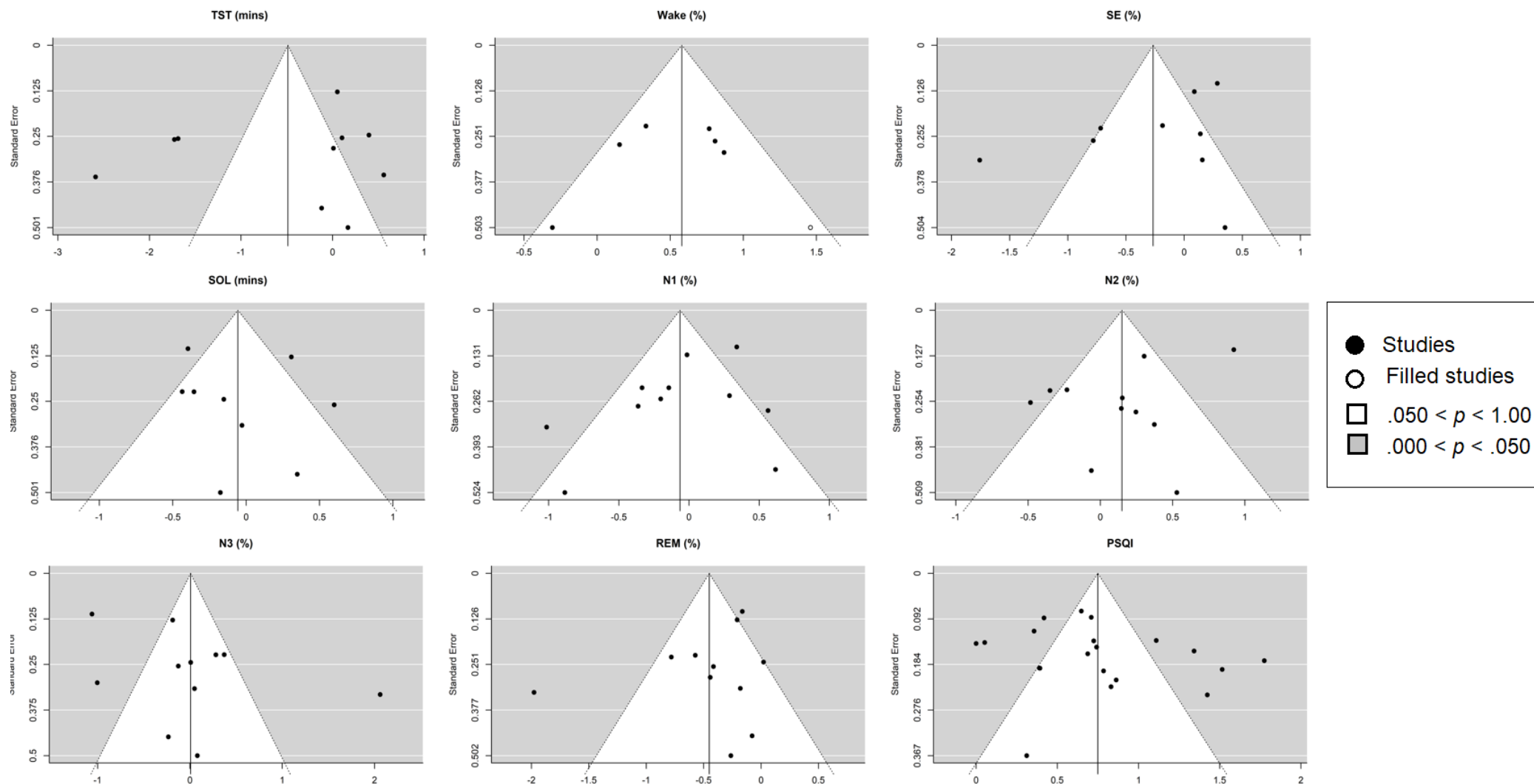
Figure 24: Trim-and-fill funnel plots for each of the meta-analyses
Trim and fill-funnel plots showing standard error (y-axis) against Hedges' *g* (x-axis*). *For wake (%) there was likely to be one study missing on the right (open circle), thus publication bias may be assumed. PSQI; Pittsburgh Sleep Quality Index; TST, Total Sleep Time; SOL, Sleep Onset Latency; SE, Sleep Efficiency; N1, non-rapid-eye-movement sleep stage 1; N2, non-rapid-eye-movement sleep stage 2; N3, non-REM sleep stage 3; mins = minutes.

3.3.3 Study Quality Results

The detailed NOS ratings for each study are included in **Table 10** and **Table 11.** The overall mean score on the NOS was 5 (SD = 1.34). The mean score on the NOS was 4.83 (range 2–7, SD = 1.43) for case–control studies and 5.44 (range 4–7, SD = 0.96) for cross-sectional studies. Four studies were categorized at very high risk of bias, 16 at high risk, and three high quality.

3.3.4 Meta-analysis Results

PSQI Score - Adults

Figure 25 displays the forest plot for the PSQI analysis in adults and controls. There was a medium effect size for the difference in PSQI scores between patients with migraine and controls (g = 0.75, p < 0.001). There was a large significant effect size for the chronic subgroups (g = 1.03, p < 0.001) and a medium significant effect size for the episodic subgroup (g = 0.63, p < 0.001). The direction of these effects indicates that patients scored significantly higher on the PSQI than controls, suggesting poorer sleep quality.

Table 10: Newcastle-Ottawa Scale scores for the case-control studies

Authors (year)	Case Definition	Selection of Controls	Definition of Controls	Comparability	ROB
Armoni et al. (2019)	*	unclear			2
Barbanti et al. (2007)	*	*		**	5
Barbanti et al. (2013)	*	*		**	5
Della Marca et al. (2006)	*			**	4
Dikmen et al. (2015)	*	*	*		4
El-Heenedy et al. (2019)	*			**	4
Engstrom et al. (2013)	*			**	4
Engstrom et al. (2014)	*	*	*	**	5
Esposito et al. (2013)	*	*		**	5
Gori et al. (2005)	*			*	2
Karthik et al. (2012)	*	*	unclear	**	5
Karthik et al. (2013)	*			*	3
Kozak et al. (2017)	*	*	*	**	6
Masuko et al. (2014)	*	*	*	**	6
Nayak et al. (2016)	*	*	*	**	6
Rafique et al. (2020)	*	*	*	**	6
Roccella et al. (2019)	*	*		unclear	2
Rockett et al. (2013)	*	*		**	5
Sengul et al. (2015)	*	*	*	**	6
Suzuki et al. (2013)	*	*	*		4
Vollono et al. (2013)	*	unclear		**	4
Walters et al. (2014)	*	*	*		4
Yon et al. (2020)	*			**	4

* and ** refer to the number of stars given based on the Newcastle-Ottawa Scale (NOS); ROB = Risk of bias

Table 11: Newcastle-Ottawa Scale scores for the cross-sectional studies

Authors (year)	Representativeness	Sample size	Non-responders	Case definition	Comparability	ROB
Chu et al. (2018)		*		*	**	4
Kristiansen et al. (2011)	*		*	*	**	5
Lin et al. (2016a)				*	**	3
Lin et al. (2016b)				*	**	3
Lin et al. (2019)		*		*	*	3
Morgan et al. (2015)	*		*	*	**	5
Sadeghniiat et al. (2013)	*			*		2
Song et al. (2018)	*			*		2
Van Oosterhout et al. (2018)	*	*	*	*		4

* and ** refer to the number of stars given based on the Newcastle-Ottawa Scale (NOS); ROB = Risk of bias
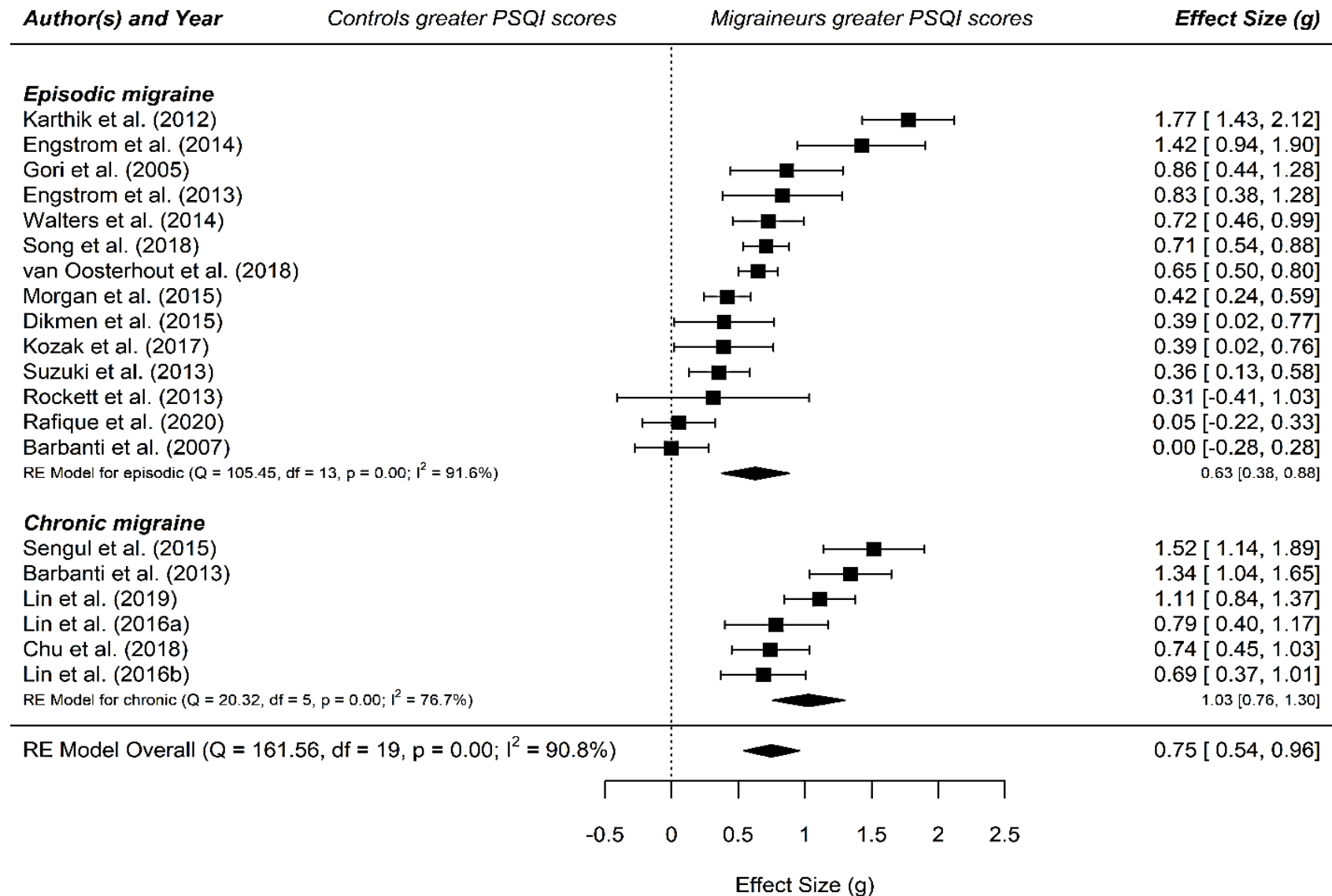
Figure 25: Forest plot of PSQI scores in migraine patients and controls
The standardized mean difference (Hedges' *g*) and confidence intervals for the difference in global PSQI scores is shown between people with migraine and healthy controls. Abbreviations: PSQI, Pittsburgh Sleep Quality Index; RE, random effects.

For the overall analysis and the episodic subgroup there was statistically significant heterogeneity as evidenced by Cochran's Q statistic (161.6 and 105.5, respectively). The chronic sub-group analysis had significantly lower heterogeneity (20.32) as measured by Cochran's Q. However, when considering the I^2 statistic the studies within the total, episodic, and chronic analyses all displayed moderate-high heterogeneity (I^2 = 90.8%, 91.6%, 76.7% respectively), suggesting that over 70% of the variability is attributable to between-study heterogeneity, over and above sampling error.

As there was moderate or greater heterogeneity, moderator analyses were conducted on the overall analysis (see **Table 12**). There was a negative moderating effect of whether the study excluded those with sleep disorders or not (Q_M = 7.40, p = 0.007, β = -0.81), suggesting that when sleep disorders were excluded the effect size is smaller. However, there was significant heterogeneity not explained by this moderator (Q_E = 114.61, p < 0.001), and as only two studies excluded those with sleep disorders this could have skewed the result. No other variables including study quality score were significant moderators.

Table 12: Results of the moderator analysis of global PSQI scores in adults

Analysis	Q_M	p	β	Q_E	p
Sleep disorders excluded	7.40*	0.007	-0.81	114.61*	<0.001
Matched controls	0.02	0.878	0.03	161.41*	<0.001
Medications excluded	0.32	0.572	0.13	160.34*	<0.001
Study quality score	0.22	0.639	-0.04	158.48*	<0 .001

* = $p < 0.05$.

Polysomnography – adults

Table 13 displays the effect sizes and heterogeneity measures for the PSG-derived sleep parameters in adults with migraine and healthy controls. There was a significant small effect size for percentage REM sleep (g = -0.22, p = 0.017). The direction of this effect indicates that the adults with migraine had less REM sleep than controls. There were no statistically significant effect sizes for the other parameters. There was significantly moderate heterogeneity as evidenced by the Q and I^2 statistic in two of the analyses (SE and wake). However, as the main measure of effect size on these parameters was not significant no moderator analyses were conducted.

Table 13: Results of the meta-analysis of sleep architecture in adults

Parameter	N Studies	N participants (C, Mi)	Hedges' g	p	Q	I²(%)
TST (mins)	6	532, 169	0.10	0.282	1.95	1.95
SOL (mins)	5	515, 152	0.24	0.100	5.49	32.56
SE (%)	4	505, 142	-0.08	0.728	10.0	73.25
Wake (%)	3	65, 63	0.58	0.053	4.48	57.89
N1 (%)	6	540, 177	0.10	0.515	10.03	44.16
N2 (%)	6	540, 177	0.11	0.458	8.33	43.10
N3 (%)	6	540, 177	-0.24	0.091	8.25*	42.99
REM (%)	6	540, 177	-0.22*	0.017	2.27	0.00

Abbreviations: C; healthy controls, Mi; migraine, PSQI; Pittsburgh Sleep Quality Index; TST, Total Sleep Time; SOL, Sleep Onset Latency; SE, Sleep Efficiency; N1, non-rapid-eye-movement sleep stage 1; N2, non-rapid-eye-movement sleep stage 2; N3, non-REM sleep stage 3; * = $p < 0.05$.

Polysomnography - pediatric

Table 14 displays the effect sizes for the PSG-derived parameters in pediatric patients with migraine. There were small significant effect sizes for wake ($g = 0.43$, $p = 0.015$) and SOL ($g = -0.37$, $p < 0.001$). There was a medium significant effect size for REM sleep ($g = -0.71$, $p = 0.025$), and a large significant effect size for TST ($g = -1.37$, $p = 0.039$). The direction of these effects indicates that pediatric patients had more wake, less REM sleep, less TST and shorter SOL than healthy controls. There was statistically significant heterogeneity in six of the analyses (TST, SE, N1, N2, N3, REM).

Table 14: Results of the meta-analysis of sleep architecture in children

Parameter	N Studies	N participants (C, Mi)	Hedges' g	p	Q	$I^2(\%)$
TST (mins)	4	146, 133	-1.37*	0.039	43.71*	1.95
SOL (mins)	4	303, 272	-0.37*	<0.001	1.32	32.56
SE (%)	5	323, 312	-0.42	0.242	48.36*	73.25
Wake (%)	3	123, 107	0.44*	0.015	3.32	57.89
N1 (%)	5	323, 312	-0.24	0.260	23.58*	44.16
N2 (%)	5	323, 312	0.19	0.448	41.68*	43.10
N3 (%)	5	323, 312	0.32	0.524	113.03*	42.99
REM (%)	5	323, 312	-0.71*	0.025	32.0*	0.00

Abbreviations: C; healthy controls, Mi; migraine, PSQI; Pittsburgh Sleep Quality Index; TST, Total Sleep Time; SOL, Sleep Onset Latency; SE, Sleep Efficiency; N1, non-rapid-eye-movement sleep stage 1; N2, non-rapid-eye-movement sleep stage 2; N3, non-REM sleep stage 3; * = $p < 0.05$.

Two of the analyses of PSG variables in pediatric patients which found significant effect sizes and displayed at least moderate heterogeneity were: TST and REM, thus a moderator analysis was conducted on these. None of the studies excluded patients with sleep disorders, so this was not included as a moderating variable. **Table 15** displays the results. For TST, both whether the study excluded patients who were on sleep-affecting medications, and whether the study included matched controls or not, were significant moderating variables ($Q_M = 19.6$, $p < 0.001$, $Q_M = 19.6$, $p < 0.001$) respectively. The test for residual heterogeneity (Q_E) was not significant for these two analyses, indicating that they are largely influencing the effect size in these studies. The direction of this moderator analysis indicated that when a study did exclude those on medication, or did include matched controls, the effect size was smaller. However,

whether the study included an adaptation night or study quality score were not significantly moderating variables ($Q_M = 0.20$, $p = 0.655$, $Q_E = 38.46$, $p < 0.001$, $Q_M = 0.92$, $p = 0.338$, $Q_E = 34.99$, $p < 0.001$ respectively). For REM sleep, there were no significant moderators of effect size and Q_E was significant for all analyses, indicating significant residual heterogeneity that cannot be explained by these moderators, and thus other variables are influencing between-study heterogeneity.

Table 15: Results of the moderator analysis of PSQI scores in children

Analysis	Q_M	p	β	Q_E	p
Adaptation night					
TST	0.20	0.655	-0.69	38.46*	< 0.001
REM	0.01	0.931	0.10	28.37*	< 0.001
Matched controls					
TST	19.60*	< 0.001	-2.51	4.75	0.093
REM	0.80	0.372	-0.70	17.73*	< 0.001
Medications excluded					
TST	19.60*	<0 .001	-2.51	4.75	0.093
REM	0.80	0.372	-0.70	17.73*	< 0.001
Study quality score					
TST	0.18	0.672	-0.30	41.87*	< 0.001
REM	0.07	0.787	0.07	31.71*	< 0.001

Abbreviations: TST = total sleep time, REM = rapid-eye-movement sleep, PSG = polysomnography

Relationship between sleep quality and migraine disability

Figure 26 shows the weighted effect size for the correlation between MIDAS and PSQI scores. There was a small non-significant effect size for the correlation between MIDAS scores and PSQI scores in patients with migraine ($z = 0.32$, $p = 0.060$), thus suggesting from this analysis that there is no relationship between sleep quality and migraine disability. There was significantly low heterogeneity between studies as indicated by Cochran's Q statistic ($Q = 28.74$, $p < 0.001$), however I^2% indicated high heterogeneity (I^2% = 91.1%), and as this statistic is more appropriate for small samples, high heterogeneity between these studies can be assumed. As the main analysis was not significant no moderator analyses were conducted.
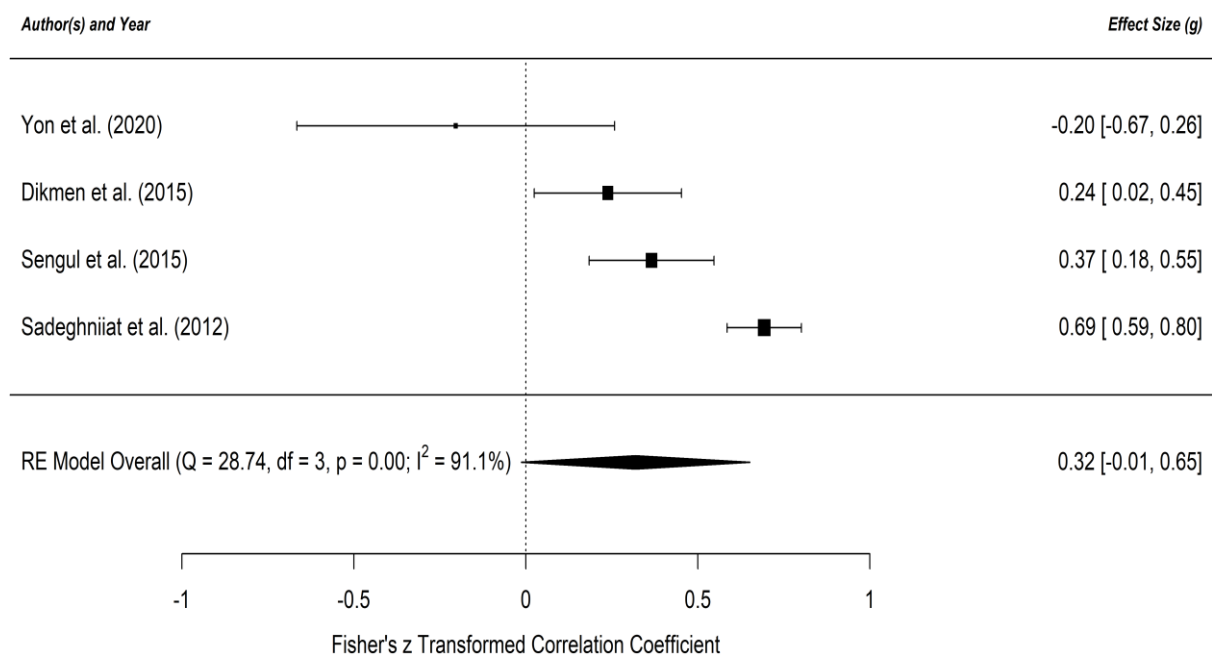
Figure 26: Meta-analysis of MIDAS and PSQI correlations
Abbreviations: RE, random effects. The Fisher's *z* transformed correlation coefficient is shown between MIDAS scores and PSQI scores in patients with migraine across four studies.

3.4 Discussion

Herein, we aimed to consolidate previous findings and establish whether there are differences in subjective sleep quality and objective sleep architecture between people with migraine and healthy individuals. We demonstrated that adults with migraine display significantly higher scores on the PSQI indicating worse subjective sleep quality than healthy controls, an effect which is larger in those with chronic rather than episodic migraine. There are differences in sleep architecture, although this was mainly evident in the pediatric population. For example, adults and children with migraine display significantly less REM sleep as a percentage of TST than healthy individuals, and this effect size is larger in the pediatric population. Pediatric patients also exhibit significantly less TST, shorter SOL, and more wake than healthy controls. There were no differences between migraine patients and healthy controls in any other sleep parameters either in adult or pediatric populations. Finally, there was no significant overall correlation between PSQI and MIDAS scores in people with migraine.

3.4.1 Subjective Sleep Quality - Adults

These findings extend the current literature by aggregating the results of multiple studies, thereby increasing power. They demonstrate that sleep quality is worse in adults with migraine than healthy individuals. Although the PSQI may be criticized as a subjective measure of sleep, it has been shown to have high test-retest reliability and high validity (Backhaus et al., 2002b). Moreover, this finding is not surprising; poor sleep is reported as an exacerbating factor for migraines in 50% of cases (Kelman & Rains, 2005), and poor sleep is commonly reported in migraine (Calhoun et al., 2006). The effect size was larger in chronic migraine patients. This is in line with previous

research where poor sleep has been shown to be an important factor in progression to chronic migraine (Bigal & Lipton, 2009).

3.4.2 Polysomnography - Adults

REM sleep was found to be reduced in adults with migraine relative to healthy individuals, aligned with previous associations between migraine and REM sleep (Dexter, 1979). For example, one study found a reduction in REM sleep the night preceding a migraine attack (Göder et al., 2001a). Furthermore, cutaneous allodynia, a prominent symptom experienced during migraine attacks, has been shown to worsen in response to REM sleep deprivation in preclinical pain models (Kim et al., 2019). This indicates a potential dysfunction in mechanisms underlying REM-NREM or REM-wake transitions in migraine patients. Interestingly, migraine is highly co-morbid with insomnia (D. Walker et al., 2023) and greater REM macro and micro arousals are observed in insomnia disorder (Feige et al., 2008, 2013). A plausible neural correlate for this is the hypothalamic orexinergic system which plays a critical role in stabilising sleep/wake transitions (Feng et al., 2020), and has been linked to migraine; or the PAG which is involved in descending pain modulation and NREM-REM sleep transitions, suggesting multiple points of intersection between sleep and trigeminal pain. Although, this is speculative, and the relationship with REM sleep is likely to be complex and bi-directional. An alternative explanation is disruption to the LC noradrenergic system, as insufficient silencing of the LC has been shown to disrupt REM sleep (Swift et al., 2018). Descending projections from the LC have been shown to regulate nociception at the spinal cord (Schwarz & Luo, 2015), suggesting a prominent role in nociception and potentially migraine, and a plausible mechanism of LC-mediated REM disruption.

There were no differences between adults with migraine and healthy controls in any other sleep parameters. This is at odds with a previous meta-analysis which found that TST, WASO and SOL were worse in those with chronic pain (including migraine) than healthy controls (Mathias et al., 2018). Although, this might be reconciled in that the number of studies they included was larger and contained heterogeneous chronic pain conditions. It is likely that with a greater number of studies, we might see significant differences in wake, TST, and SOL in adults.

3.4.3 Polysomnography - Children

Pediatric patients also displayed a lower percentage of REM sleep as well as shorter TST and more wake, in line with previous findings in pediatric patients with chronic pain (Palermo et al., 2007). The migraine patients also displayed shorter SOL than controls. This is suggestive of children with migraine operating at higher sleep pressure due to being chronically sleep deprived (perhaps due to headache, behavior, or both) hence their shorter SOL times. Alternatively, children with migraine may be biologically sleepier than their healthy counterparts. Their shorter SOL is at odds with findings from actigraphy (Bruni et al., 2004) and questionnaire-based studies (Spierings & Hoof, 1997) in pediatric migraine patients which found a longer SOL in patients compared to healthy controls. Although, actigraphy and self-report data are less accurate than PSG, particularly for SOL (Sivertsen et al., 2006). However, it should be noted that the current effect size is small and generated by a small number of studies.

The lack of any significant difference in NREM sleep, including N3, in both adults and children with migraine compared to controls is noteworthy, although this data come from macroscopic sleep architecture rather than quantitative EEG analysis. Deficits in NREM sleep can be compensated for by an increase in sleep intensity rather

than duration as is the case typically for REM sleep (Borbély & Neuhaus, 1979), which would not be apparent with macroscopic scoring. These differences could instead be captured by observing delta power changes in NREM sleep. Furthermore, specific neural oscillations during NREM sleep such as sleep spindles and hippocampal ripples may be altered in migraine, however, there remains a paucity of evidence in this area.

3.4.4 Migraine Disability

The lack of a significant correlation between PSQI and MIDAS scores in adults is surprising given that previous studies report significant correlations between migraine-related disability and sleep disturbances (Rodríguez-Almagro et al., 2020), and altering sleep (e.g. by switching back to day shifts from night shifts) can have a profound effect on migraine-related disability scores (Sandoe et al., 2019). This may reflect the small number of studies and the high heterogeneity between them. Three of the studies in this analysis found significant positive correlations (Sadeghniiat et al., 2013), and one found a large negative correlation (Yon et al., 2020). Studies with a small n can lead to inflated effect sizes (Slavin & Smith, 2008). Indeed, the study which found a negative relationship had an n of 21 (Yon et al., 2020), compared to 332 for one of the larger studies, thus potentially skewing the results towards insignificance. There was also evidence of publication bias within this analysis. Although caution should be taken when interpreting the results as despite meta-analysis being theoretically sound on a small number of studies, Egger's test can produce false positives.

3.4.5 Limitations & Future Directions

A critical consideration is that many people with migraine are given prophylactic treatments which can affect the sleep cycle. Due to the difficulties when recruiting drug-naïve patients, most studies did not exclude patients on medications. For many

studies it was impossible to deduce whether patients on medication were excluded. However, when this factor was included as a moderating variable in the pediatric analysis for TST and REM sleep, studies which excluded those on medications had a smaller effect size for TST than those which did not, suggesting that the patient's medication may be contributing to differences in TST. Indeed, β-blockers which are used in the prevention of migraine may potentially reduce TST (Betts & Alford, 1983). See **Table 16** for an overview of commonly used headache medications and their potential impact on sleep architecture. Nonetheless, significant residual heterogeneity remained between studies which could not be explained by this moderator, suggesting that other factors are at play. Importantly, medication exclusion did not impact REM sleep. Although, it is unclear whether medication was driving the REM sleep effect seen in adults as no moderator analysis was conducted on this.

Table 16: Medications for headache treatment and their potential impact on sleep

Medication	Use in migraine	TST	NREM	REM	Wake	Arousals	REM SOL	SOL	SE	Somnolence	Miscellaneous	References
Tricyclic Antidepressants												
amitriptyline	preventive	↑		↓		↓	↑	↓			REM rebound, improved sleep continuity, reduced REM atonia	DeMartinis et al. (2007); Wilson & Argyropoulos (2005); Mayers & Baldwin (2005); Hoque & Chesson (2010)
Anti-convulsants												
topiramate	preventive								↑		Mixed evidence: no difference in sleep	Bonanni et al. (2004); Diener et al. (2004); Brandes et al. (2004)
carbamazepine	preventive					↓		↓	↑			Horst et al. (1994); Riemann et al. (1993)
sodium valproate	preventive										Delayed circadian rhythm	Johansson et al. (2011)
gabapentin	preventive		↑			↓	↓					Foldvary-Schaefer et al. (2002); Hindmarch et al. (2005)
NSAIDs												
ibuprofen	acute				↑	↑			↑			Gengo et al. (2006)
aspirin	acute				↑	↑						Murphy et al. (1994); Horne et al. (1980)

		TST	NREM	REM	SOL	SE	SWS	Notes	Reference
Triptans									
sumatriptan	acute					↑			Derry et al. (2012)
zolmitriptan	acute					↑			Law et al. (2013)
Serotonin antagonists									
trazadone	preventive	↑	↑						Kaynak et al. (2004); Ware and Pittard (1990); Wichniak et al. (2021)
methysergide	preventive		↑	↓					Mendelson et al. (1975)
Miscellaneous									
melatonin	preventive					↑			Hughes et al. (1998)
benzodiazepines	preventive		↓	↓				Increased stage 2 NREM	de Mendonça et al. (2023)
caffeine	acute		↑			↑		SWS increase only in second sleep cycle	Paterson et al. (2009)
fluoxetine	preventive			↓				Reduced REM atonia	Wilson & Argyropoulos (2005); Hoque & Chesson (2010)
β-blockers	preventive			↓	↑	↑		Nightmares, vivid dreams	Pagel & Helfer (2003); Betts & Alford (1983)
calcium channel blockers	preventive							Changes in NREM sleep spindles	Ayoub et al. (2013)
paracetamol	acute		↓			↑	↓		Onen et al. (2005)

NSAIDs = non-steroidal anti-inflammatory drugs; TST = total sleep time; NREM = non-rapid-eye-movement sleep; REM = rapid-eye-movement sleep; SOL = sleep onset latency; SE = sleep efficiency; SWS= slow-wave sleep.

These results do not provide evidence for a direct functional relationship between migraine and sleep regulation. Are people with migraine experiencing poor sleep due to the pain caused by attacks, or are they experiencing attacks due to poor sleep? Alternatively, a third variable could underly this relationship (e.g. genetic alteration). The studies in this analysis did not report whether the patients typically experience their migraine attacks during sleep itself, despite two thirds of migraine sufferers reporting experiencing them during sleep (Kelman & Rains, 2005). Only a handful of studies reported whether the PSG was conducted in the ictal or inter-ictal period, despite this being shown to have a considerable effect on objective measures of sleep (Bjørk & Sand, 2008). If this were to affect the results however, we might have expected to see a difference in other sleep parameters in both the adults and pediatric populations. As we only see the decrease specific to REM sleep and no other sleep stages, this implies a specific dysfunction in the mechanisms that underlie REM sleep.

Alternatively, the reduced REM sleep could reflect the finding that many migraine attacks occur in the early hours of the morning - the portion of the night where REM sleep dominates, hence curtailing REM opportunity. However, two of the PSG studies in the current analysis had a population of migraine patients with predominantly sleep-related attacks (Engstrøm, Hagen, Bjørk, Gravdahl, et al., 2013; Vollono Catello et al., 2013) and neither study reported differences in REM sleep between SRM, NSRM or controls. This implies that the decrease in REM sleep we see here is not necessarily due to arousals from REM sleep during an attack.

The observed decrease in REM sleep could also reflect the high co-morbidity of major depressive disorder in patients with migraine (Zhang et al., 2019). Whilst patients with depression may present with an increase in the proportion of REM sleep and enhanced REM density, many treatments for depression such as selective-

serotonin re-uptake inhibitors, serotonin antagonists, and tricyclic antidepressants may reduce the proportion of REM sleep (Wilson & Argyropoulos, 2005) which could explain the current finding. However, this is unlikely as most of the included studies excluded those on medications other than triptans and NSAIDs and whilst not investigated in adults, in children medication exclusion was not found to be a significant moderator of the REM sleep result.

Moreover, six of the included studies did not include an adaptation night to the sleep laboratory. The 'first night effect' has been shown to particularly affect measures of REM sleep (Lorenzo & Barbanoj, 2002). However, the moderator analyses in pediatric patients found that whether the study included an adaptation night or not was not a significant moderator of the overall effect size for REM sleep or TST.

The disparity between the large effect size seen with the subjective measure of sleep and the lack of significant difference between migraine patients and controls with all but one of the objective sleep physiology parameters in the adult population is not unexpected. Global PSQI scores have been shown not to significantly correlate with sleep variables as measured via PSG (Grandner et al., 2006). As we did not analyze PSQI scores in the pediatric population, it is not clear if pediatric patients also experience altered subjective sleep quality. This being said, the PSQI has been shown to have limited utility in pediatrics (Erwin & Bashore, 2017).

In addition, many of the studies were retrospective studies of patients who have previously been referred to the sleep clinic with non-specific sleep complaints, necessitating PSG assessment, suggesting the patients with migraine referred may already report underlying sleep complaints. Few of the studies mentioned prior sleep history in relation to PSG, and for subjective sleep quality those which did exclude sleep disorders had a smaller effect size than those which did not, suggesting that poor

sleep quality may be attributable to undiagnosed concurrent sleep disorders. However, this is unlikely to be the case as the heterogeneity between studies was not fully explained by this variable. This residual heterogeneity may reflect differences in pre-recording sleep history, differing recording environments, techniques and equipment, as well as differences in inter-scorer scoring concordance (Collop, 2002).

Moreover, one concern is that many studies did not record whether patients could nap on the day of PSG assessment, yet it is plausible that migraine patients nap more than healthy controls to relieve their migraine symptoms (Ong et al., 2023), meaning that during the nocturnal sleep there is less REM sleep. However, if this were the case SOL would be expected to be longer in migraine patients than controls as recent evidence suggests (Dawson et al., 2019), yet SOL was shorter in the pediatric patients in the present analysis.

3.4.6 Clinical Implications

These findings highlight that consideration of patient sleep should play an integrated role in the assessment and treatment of migraine. Clinicians may consider prioritizing behavioral sleep interventions (especially in children with migraine) as well as considering sleep when prescribing medication. Recent studies have demonstrated the utility of sleep behavioral interventions in reverting chronic to episodic migraine (Calhoun & Ford, 2007), which may suggest a potential role of instigating similar interventions earlier in the natural history of the disorder to mitigate the risk of conversion from episodic to chronic migraine. Interestingly, a recent study followed patients treated with erenumab (CGRP-receptor MAb) and they displayed significant improvements in objective and subjective sleep quality, suggesting that by treating migraine, concurrent improvements in sleep may be observed (Pellitteri et al., 2022). The relevance of REM sleep and its modulation for

migraine and migraine treatment is again emphasized by this meta-analysis. The relationship is likely to be complex and related to sleep (and indeed REM) homeostasis, rather than related to any absolute proportions of REM sleep.

3.4.7 Conclusions

People with migraine report poorer subjective sleep quality than healthy individuals, an effect larger in those with chronic migraines. Adult patients exhibit significantly less REM sleep compared to healthy controls, whereas pediatric patients also show significantly reduced sleep time, faster sleep onset and more wake than controls. The interplay between migraine and sleep is likely to be highly complex and remains poorly understood. However, the findings of this meta-analysis highlight that assessing and treating sleep in migraine may be useful as an integrated part of headache treatment. While offering significant insight into how sleep is relevant to migraine, this study also highlights the limitations of drawing conclusions from small case-controlled PSG studies of patients with migraine given the significant number of confounds and heterogeneity involved. Furthermore, it should be acknowledged that for some sleep variables there were a limited number of studies and caution should be taken when drawing conclusions between sleep and migraine. Future studies should analyse the interplay between migraine and subjective sleep factors at large-scale population-based levels, as well as also using a more population-based approach to sleep physiological studies, performed in a standardized way, to minimize variability where possible.

Chapter 4: Predicting Migraine Attacks with Sleep Variables

The work in this chapter is published as a peer-reviewed publication:

Stanyer, E.C., Brookes, J., Pang, J.R. et al. Investigating the relationship between sleep and migraine in a global sample: a Bayesian cross-sectional approach. *J Headache Pain* **24**, 123 (2023). https://doi.org/10.1186/s10194-023-01638-6

The associated data analysis scripts are available on GitHub.

4.1 Introduction

In the previous chapter we investigated subjective and objective sleep in migraine patients and uncovered poorer subjective sleep quality and reduced REM sleep in patients compared to healthy controls. However, from this meta-analysis we do not know whether poor sleep may be involved in precipitating migraine attacks or whether simply experiencing attacks (the pain phase) causes disrupted sleep.

As noted in the introduction (see section 1.6), previous studies have aimed to disentangle this relationship and establish whether sleep variables can predict occurrence of a migraine attack, or whether attacks disrupt sleep. For example, research demonstrated that hours slept on the previous night was predictive of pain (Edwards et al., 2008) and migraine (Houle et al., 2012) the next day. Conversely, other studies have reported that sleep fragmentation, but not reduced hours slept, increases pain (Smith et al., 2007), and is associated with higher odds of having a migraine attack the next day (Bertisch et al., 2019). To our knowledge however, no studies have investigated the impact of migraine attacks specifically on subsequent sleep. Although, numerous studies have demonstrated that pain generally during both the day and night results in poor subsequent sleep including reduced TST, frequent awakenings, and sleep quality (Bjurstrom & Irwin, 2016; Keilani et al., 2018; Marty et al., 2008; Mulligan et al., 2015; Wylde et al., 2011). Therefore, it is likely that the pain experienced during migraine attacks would also disrupt sleep.

Moreover, jet-lag and shift work (Ahn & Goadsby, 2013; Leso et al., 2020) as well as changes in the surrounding environment (e.g. weather, light levels; Hoffmann et al., 2015) are key triggers for migraine, Thus, it may not be disrupted or reduced sleep that precipitates attacks, but instead the deviation from an individual's usual sleep. This is difficult to establish in clinical sleep studies as often participants will spend just one

147

or two nights in the laboratory. In support of this, research has shown that shift work is associated with a higher likelihood of developing chronic pain conditions such as lower back pain (Zhao et al., 2012).

Whilst the above studies have attempted to elucidate the bidirectional relationship between sleep and migraine, many have small sample sizes (Houle et al., 2012; Vollono Catello et al., 2013), focus solely on one specific population at one time point (Engstrøm, Hagen, Bjørk, Stovner, et al., 2013; Karthik et al., 2013), or include biased samples (e.g. patients reporting to sleep clinics for co-morbid sleep complaints), and they provide conflicting findings. However, it is important to understand the directionality of this relationship, in order to explore mechanisms and identify novel treatment targets.

4.2 Aims and Hypotheses

Therefore, the current study aimed to reconcile this disparity and investigate the relationship between sleep and migraine within a longitudinal, global sample using self-reported measures collected from a smartphone application. Moreover, we aimed to tease apart causality and establish whether sleep disruption predicts occurrence of attacks or alternatively whether attacks predict disrupted sleep. This analysis differs from the previous literature as it avoids issues with retrospective reporting, as users report migraine attacks as and when they happen, and sleep measures are automatically detected by the application. Moreover, this is the first study to directly investigate the impact of migraine attacks on subsequent sleep.

As the previous literature is conflicting, we hypothesise that both a greater number of sleep interruptions (Bertisch et al., 2019), and shorter sleep duration the night before, will predict the occurrence of a migraine attack the next day (Houle et al., 2012). Secondly, in line with changes in sleep timing as a key trigger for migraine

(Leso et al., 2020) we hypothesise that deviation from average monthly sleep duration the night before, will predict the occurrence of an attack the next day. Lastly, predicated on research reporting disrupted sleep following other pain conditions (Bjurstrom & Irwin, 2016; Wylde et al., 2011), we hypothesise that both experiencing a migraine attack and the pain intensity associated with that attack will predict a reduction in hours slept compared to average sleep on the evening on which the attack begins.

4.2.1 Approaches to Sleep Measurement

Before outlining the methodology of the study, it is important to provide an overview of the methods used to track sleep, and therefore put the current smartphone measurement into context. As detailed in **Chapter 3**, the gold standard of sleep tracking both in the clinic and for research purposes, is polysomnography (PSG). Alternatively, wrist actigraphy (e.g. Actiwatch) is a commonly used, non-invasive method of determining sleep-wake patterns, which relies on movement tracking using accelerometers (Sadeh, 2011). There are various commercial actigraphy devices which have been developed (e.g. Garmin; Grandner & Rosenberger, 2019), which also integrate heart rate or pulse oximetry measures (e.g. Fitbit). Whilst these wrist-worn devices may not be very accurate for differentiating lighter NREM sleep stages (Mantua et al., 2016), they are thought to be aligned with PSG and research-grade actigraphy for TST, SOL, and wakefulness estimates (Chinoy et al., 2021; Marino et al., 2013; Mouritzen et al., 2020; Svensson et al., 2019).

Alongside PSG, wrist-worn actigraphy devices are the most typically used in research. However, there are a variety of other commercial methods used to estimate sleep which rely on sensing movement, respiration, heart rate, pulse rate, or a combination. This includes armbands (Roane et al., 2015), ankle bands (Bobovych et

al., 2020), ballistocardiography bed sensors (Yi et al., 2019), infrared non-contact movement sensors (Schade et al., 2019) placed at the individual's bed side, and rings (de Zambotti et al., 2019). Questions have been raised regarding their validity (Kholghi et al., 2022; Tuominen et al., 2019). For example, these devices are correlated with PSG but show poor alignment on measures of sleep stages (Kim et al., 2022). However, they are thought to be useful for estimating TST, and binary sleep-wake measures (Shin et al., 2015).

Although some of these methods are non-invasive, they still require a cost and nightly set-up by the individual. Smartphone applications on the other hand require minimal input from the user (Ong & Gillespie, 2016), and are often freely available (e.g. Sleep Cycle). These applications use accelerometers, audio recording, and usage of the mobile device to detect sleep/wake and respiration rate. Research shows that most smartphone applications used to track sleep meet or exceed the accuracy of wrist-worn actigraphy devices (Fino & Mazzetti, 2019), with particularly high accuracy for binary sleep-wake detection (Bhat et al., 2015). Therefore, for detecting overall TST, smart-phone-based sleep trackers are thought to be valid.

4.3 Methods

4.3.1 Design and Inclusion Criteria

This study followed the STROBE reporting guidelines (see **Appendix 5**). This was a retrospective cross-sectional study on self-reported data gathered from the Migraine Buddy mobile application (Healint Pte. Ltd., https://healint.com). Data were collected between 30th June 2021 and 31st December 2021. Only application users which were active for at least six months and reported an activity in each month during the collection period were included. Six months' worth of data was chosen as it avoids issues with seasonal migraine triggers and circannual periodicity (Alstadhaug et al.,

2007). Users were required to have signed up to the application before the 7th January 2021 and had to have 25 or more days of user-confirmed sleep records in each month during the period. For data analysis purposes, we excluded users with <8 and >25 attacks per month on average (see section 4.3.4 for more details). There were no restrictions on the geographical location, age, or gender of the participants and these were optional for the participant to report. No ethical review board was required due to there being no user-identifiable information collected or stored. When users signed up to the application they agreed to the terms and conditions of the Healint Pte. Ltd. policy of data collection and disclosure.

4.3.2 Data Transformation and Pre-processing

The following self-reported variables were gathered by the application: demographics (if reported: age, gender, country), start and end times of each sleep episode, and start and end times of each reported migraine attack, whether an attack was thought to be triggered by menstruation by the participant (yes/no), the highest pain intensity for each migraine attack (visual analogue scale 0-10), and the symptoms experienced with each attack. It is important to note that users could either report the attack as and when it started, or retrospectively. Thus, associated pain measurements could also be retrospective, and these could be updated during an attack or after. For the purposes of demographic reporting, users were categorised by age as follows: young adults <40 years, and older adults ≥40 years. Participants could choose from a range of default symptoms for each attack or alternatively use a free-text response to enter a non-default symptom.

To account for the different time zones in the dataset, times (sleep and migraine attack start and end times) were converted from Coordinated Universal Time (UTC) to the user's local time zone. The mean number of attacks per month over the six

months for each participant was computed from the start and end times of attacks. For the purposes of demographic reporting, the percentage of attacks which were reported to be triggered by menstruation was calculated for each individual.

4.3.3 Measurement of Sleep

The measurement of sleep by the application is based upon when a user picks up their mobile phone during the night, thus if they do not pick up their phone, they are assumed to be asleep. The user can then confirm their estimated hours slept detected by the application the following morning, and only confirmed sleep records were included in the dataset. As noted in the introduction, this measurement of sleep is not the gold standard, however, it is thought to be valid for detecting general sleep and wake parameters (Bhat et al., 2015; Fino & Mazzetti, 2019), and in the current study we were interested in overall prediction of sleep duration and fragmentation.

The number of hours slept was calculated from the start and end times and number of unique sleep episode identifiers. Based on the start time of the sleep, it was assigned to a night, and defined as the same night if a sleep started before 08:00 AM. This aims to avoid two sleep episodes (e.g. one beginning at 11:59 and another at 00:01) being classified as a different night's sleep. Based on this information, if multiple sleep episodes were recorded on one night this was counted as a sleep interruption. To generate the number of interruptions variable we used the number of separate sleep episodes per night minus one. The time in bed variable was calculated as the difference between the start time of the first sleep episode of the night and the end time of the final sleep event for that night. Wake time was calculated as the time in bed minus the number of hours slept. Sleep efficiency was calculated as time asleep divided by time in bed multiplied by 100 based on convention. To calculate deviations from monthly mean hours slept, for each user we computed a Z-score of their total

hours slept and took the absolute value of the Z-score of an individual's deviation, and for each night calculated how many standard deviations away from the mean the sleep duration was.

It is important to note that whilst users could not directly report naps in the application, they were unlikely to have been counted in the sleep duration measurements. The app uses automatic sleep detection; however, users set parameters for when they want their automatic detection to activate and deactivate e.g. 22:00-08:00. Thus, the app would not automatically detect naps during the day, as well as sleep that was earlier or later than these times. However, users must confirm their sleep the next morning thus they could adjust if their sleep was outside these pre-set times.

4.3.4 Measurement of Migraine

A similar approach was taken to classify migraine attacks. A migraine attack and its duration was classified based on the user self-reported start and end time. Users who reported on average more than 25 migraine attacks per month were excluded (n = 61). If users had daily attacks, it would be difficult to establish attack onset and fit a predictive model. Similarly, for the Bayesian analysis, we removed users reporting <8 attacks per month due to the difficulties fitting a predictive model with infrequent attacks. We removed any individual migraine attacks which were greater than 96 hours, as typical attacks last between 4 and 72 hours (The International Classification of Headache Disorders, 3rd Edition, 2018) and these were likely to be data entry errors. As migraine attacks typically span multiple days, we calculated the duration of migraine experienced on each day as well as the date that the attack started. For example, if it started at 20:00 on one day but ended at 16:00 the next day attack duration would be classified as four hours on day one, and 16 hours on day two.

Importantly, users simply stated that an attack started. It is likely that this refers to the headache pain phase, however we do not have information on how user's self-defined when attacks began and ended. For all variables the mean and standard deviation were computed.

4.3.5 Bayesian Modelling

We used Bayesian estimation techniques to infer distributions of possible parameter values in each model. We chose a Bayesian approach rather than traditional frequentist statistics as this avoids issues surrounding the interpretation and arbitrary nature of p-values and statistical significance (Matthews, 2001; Amrhein et al., 2019; Wasserstein & Lazar, 2016). Bayesian linear regression allows quantification of evidence in favour of, or against, a substantive model compared with a baseline model (Baldwin & Larson, 2017; Gadie et al., 2017).

To establish whether sleep variables could predict occurrence of a migraine attack on a given day, we created a generalized linear model with a constant plus three predictor variables: deviation from an individual's mean sleep duration (Z-score), number of sleep interruptions, and total hours slept, and whether an attack occurred or not (yes/no) as the binary dependent variable. Conversely, we created a linear model supposing that total hours slept on a given night could be predicted from a constant plus two predictor variables: occurrence of an attack (0 or 1) and pain intensity of an attack (0 – 10).

We used Bayesian inference to estimate the posterior distribution of every parameter value, which quantifies the relationship between that predictor variable and the outcome variable. In both models, parameter values were estimated per participant, and assumed to come from a normal distribution, with the mean and standard deviation of the distribution also being estimated.

We implemented these analyses using the *BRMS* package (Bürkner, 2017) in *R*. *BRMS* uses Stan (Stan Development, 2018) and implements a Hamiltonian Markov Chain Monte Carlo algorithm. We estimated the posterior distribution P(Parameters | Data) using the No-U-Turn algorithm (Hoffman & Gelman, 2011) implemented in *BRMS*. For the first model with attack occurrence as the outcome variable, Bernouli was used as the family function, and for the second where hours slept was the outcome variable, the default Gaussian was used. Four separate chains of 4,000 samples were taken from the posterior distribution, the first 1,000 samples were discarded as warmup samples. We verified the chains did not diverge using trace-plots after sampling, and $\hat{R}$ values were close to 1.000 in both models for all parameters. Parameters' priors were all set to be uninformative normal distributions with mean = 0 and standard deviation = 100.

We computed 95% highest density intervals (HDIs) on population mean parameters, plotted posterior distributions as a density plot, and used the positioning in relation to 0 to assess the likelihood of a variable being a significant predictor. Posterior distributions are a probability distribution which represent our updated beliefs (uncertainty or certainty) about the parameter/s after having seen the data, combined with what we knew before the data were collected (the prior belief). In this case, as we used uninformative priors, the posterior distribution is driven entirely by the data. Posterior means of the β-value estimates for each model were computed.

4.4 Results

4.4.1 Demographics

There were 11,166 app users in the dataset. We removed any users which reported their age as <18 years as sleep architecture can change with age (Dorffner et al., 2015) and we were interested in studying the relationship between sleep and migraine in

adults. As the app does not require reporting of age or gender, of the 11,086 users, 7239 did not report their age, leaving age data for 3847 users. The mean age of the 3847 users which reported it was 41.21 years (range = 18-81 years old, SD = 11.50) (see **Figure 27**). Users which did not report their age or demographics were not excluded from the analysis, as these variables were not included in any analyses but simply used for demographic reporting. There may have been users under 18 years of age which did not disclose their age, therefore it is possible that some users under 18 were included in the analysis. When categorizing adults as younger (<40 years of age) and older (≥40 years), there were 1729 younger adults and 2118 older adults.

Of the 11,086 users, 5911 self-reported as female (53.32%), 1276 as male (11.51%), and 3899 did not report their gender (35.17%). This demographic data mirrors that seen in epidemiological studies (Steiner & Stovner, 2023; see section 1.1.1) with a much higher prevalence of migraine in females, and a peak in prevalence around 30-40 years of age (Ripa et al., 2015).

Users were reportedly based in 99 different countries (see **Figure 28**) with most of the app users residing in the United States of America (39.34%), Japan (10.54%), Germany (6.68%), United Kingdom (6.43%), and France (6.29%) at the time at which they signed up to the app. The sleep and migraine variables according to geographical region are shown in **Figure 29-31.**

4.4.2 Sleep and Migraine Attack Characteristics

The descriptive statistics for the sleep and migraine measures according to age group and gender are shown in **Tables 15-16**, respectively.
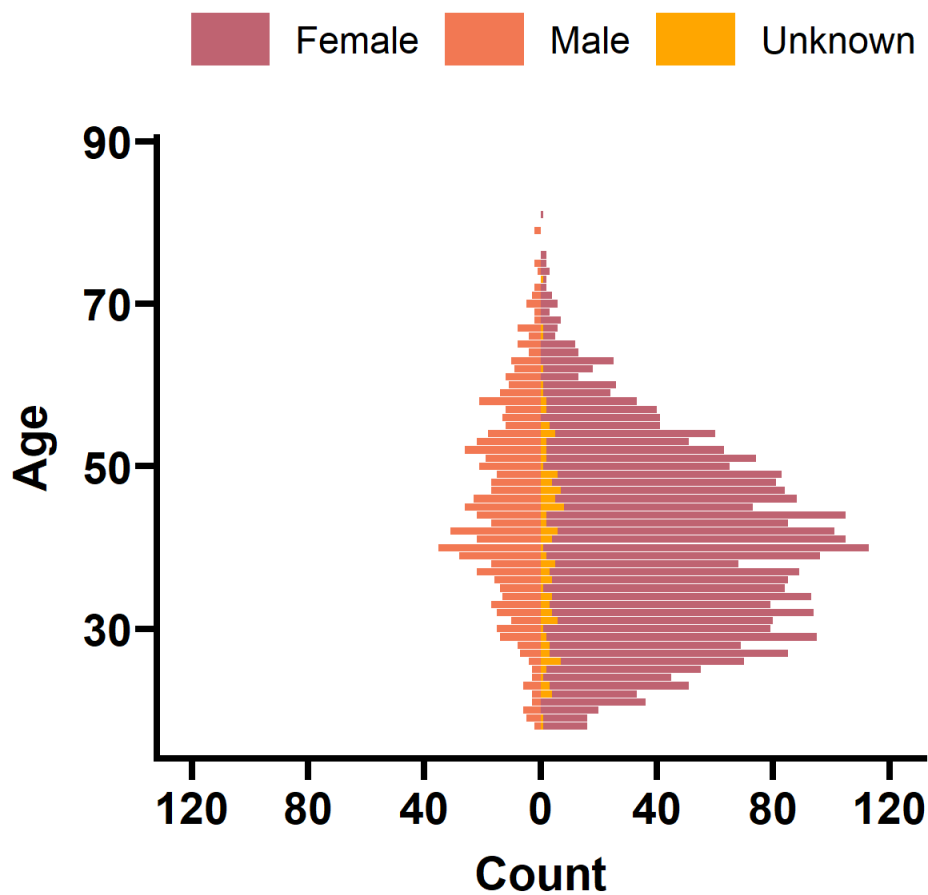
Figure 27: Population pyramid of users in the dataset
The population of users mirrors that seen in epidemiological studies (Steiner & Stovner, 2023) with a much higher prevalence of migraine in females, with a peak around 30-40 years of age (Ripa et al., 2015).
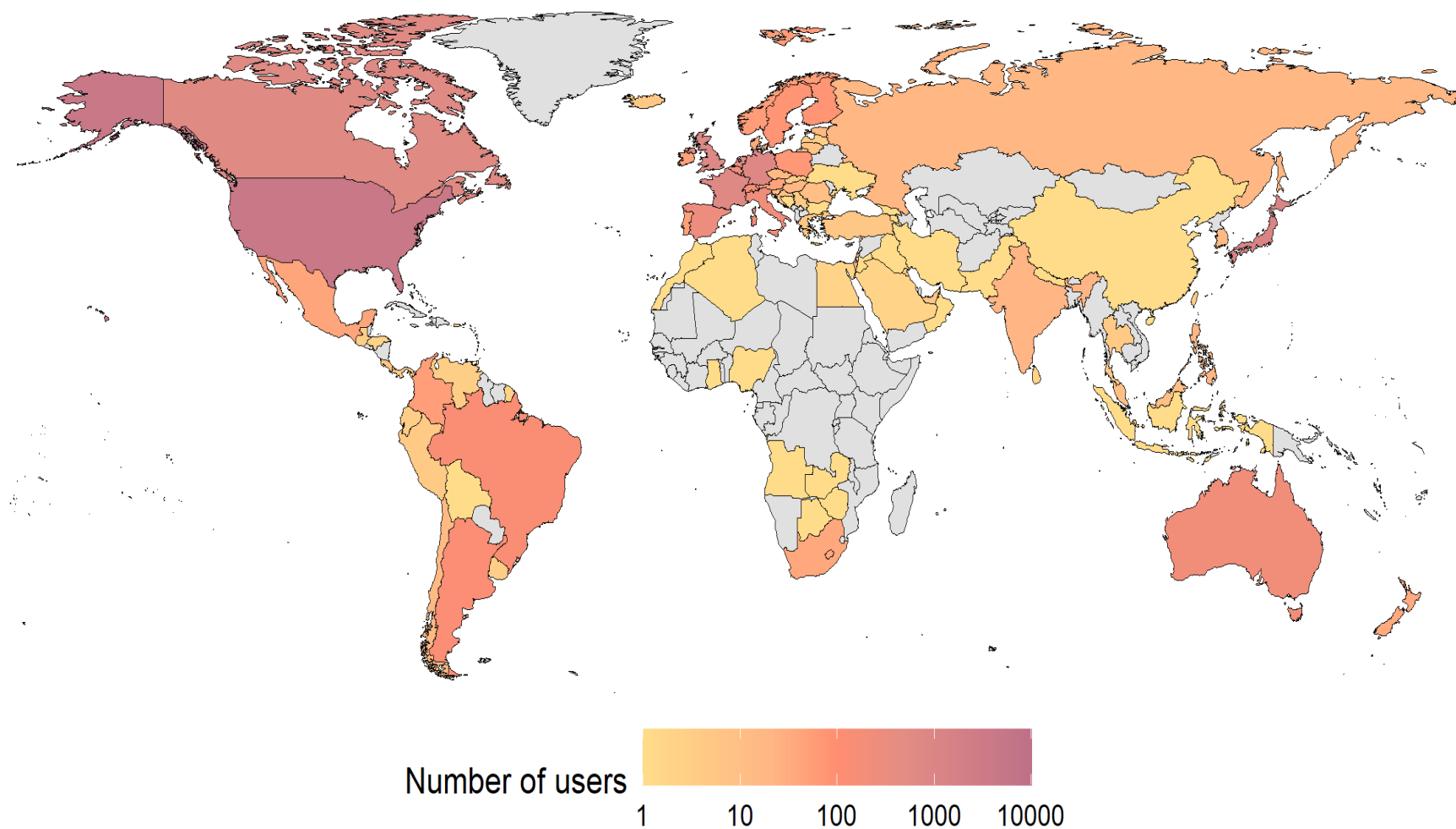
Figure 28: Geographical distribution of users in the dataset
Choropleth map showing the distribution and number of users across the world, with most users based in the USA, Canada, Japan, Germany, UK and France. Countries in grey are those without any users in the current dataset.
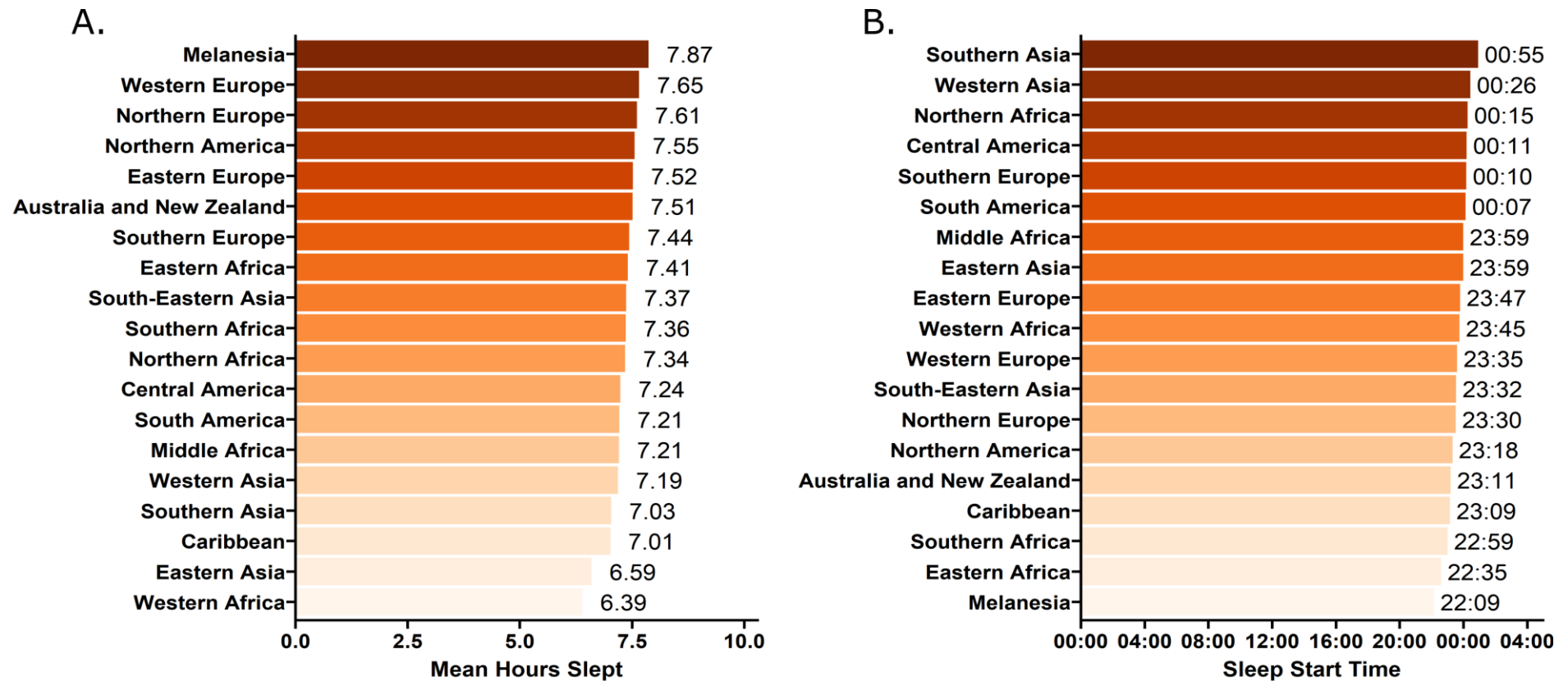
Figure 29: Sleep characteristics according to geographical region
A) Mean hours slept according to region, with the shortest sleep duration (6.39 hours) in Western Africa and the longest duration in Melanesia (7.87 hours). **B)** Mean sleep start time according to region. The latest sleep onset time was in Southern Asia (00:55) and the earliest was in Melanesia (22:09).
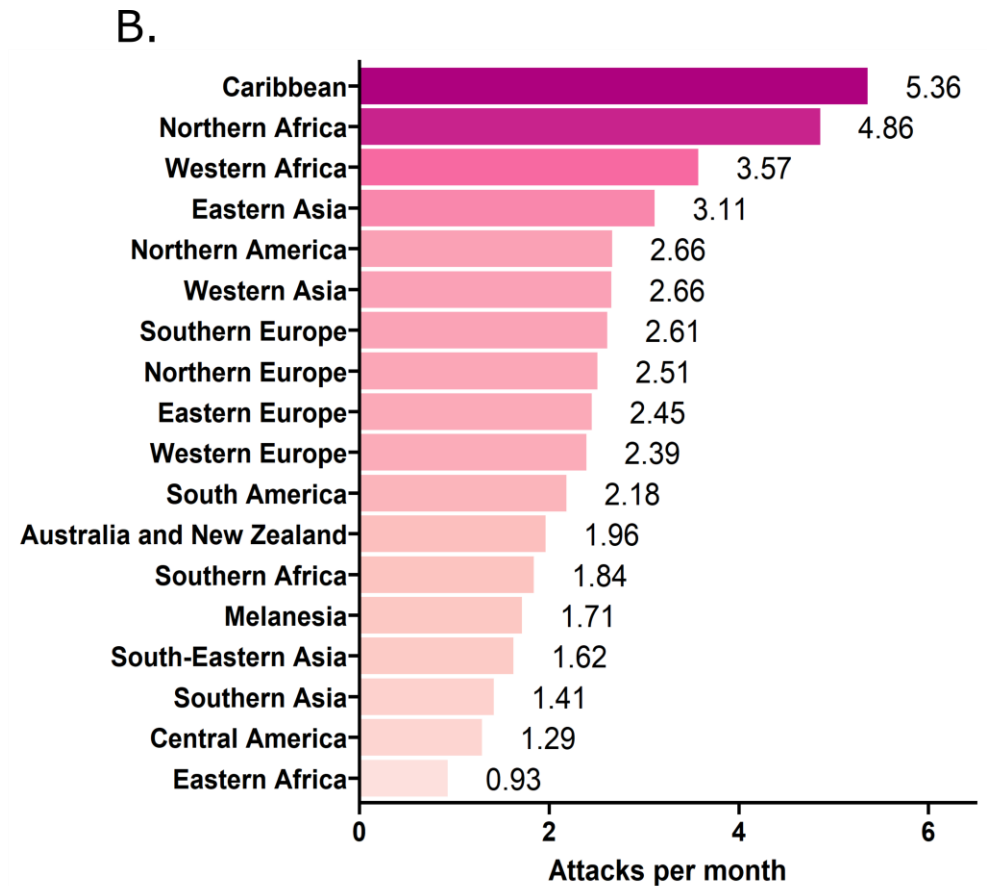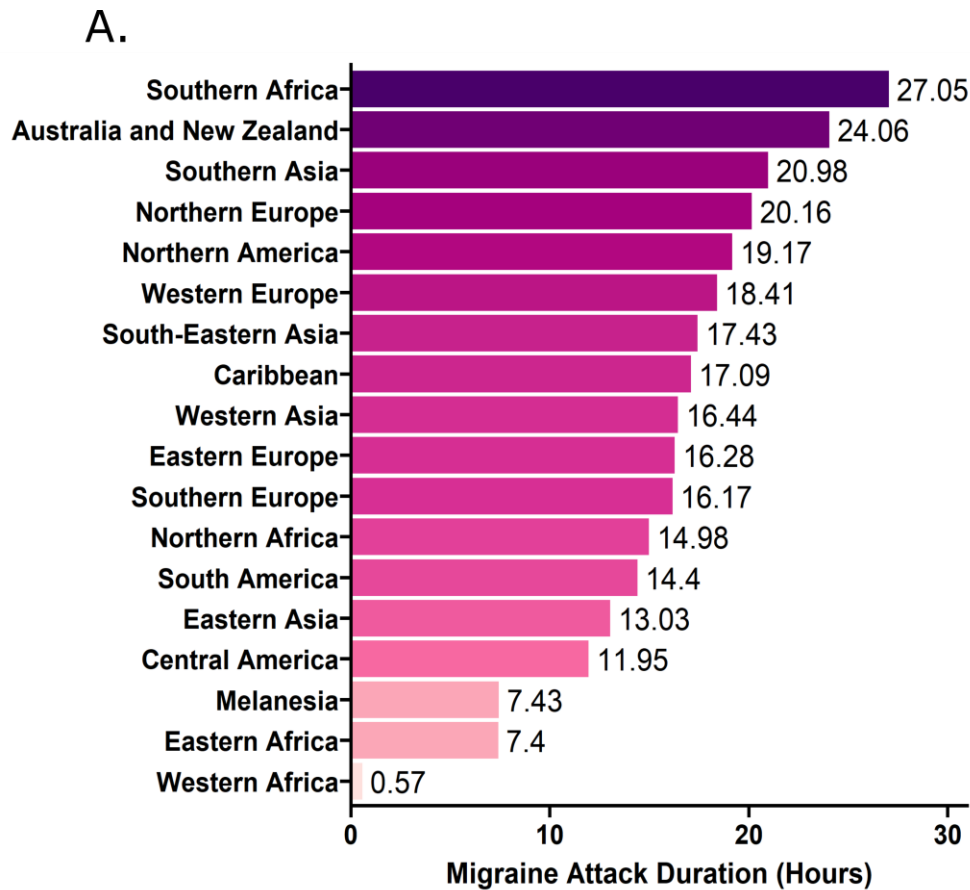
Figure 30: Migraine characteristics according to geographical region

A) Migraine attack duration according to region. The longest self-reported migraine attack durations tended to occur in Southern African, with the shortest durations in Western Africa. **B)** The number of attacks per month according to region. Users in the Caribbean reported the most attacks per month compared to those in Eastern Africa reporting the least.
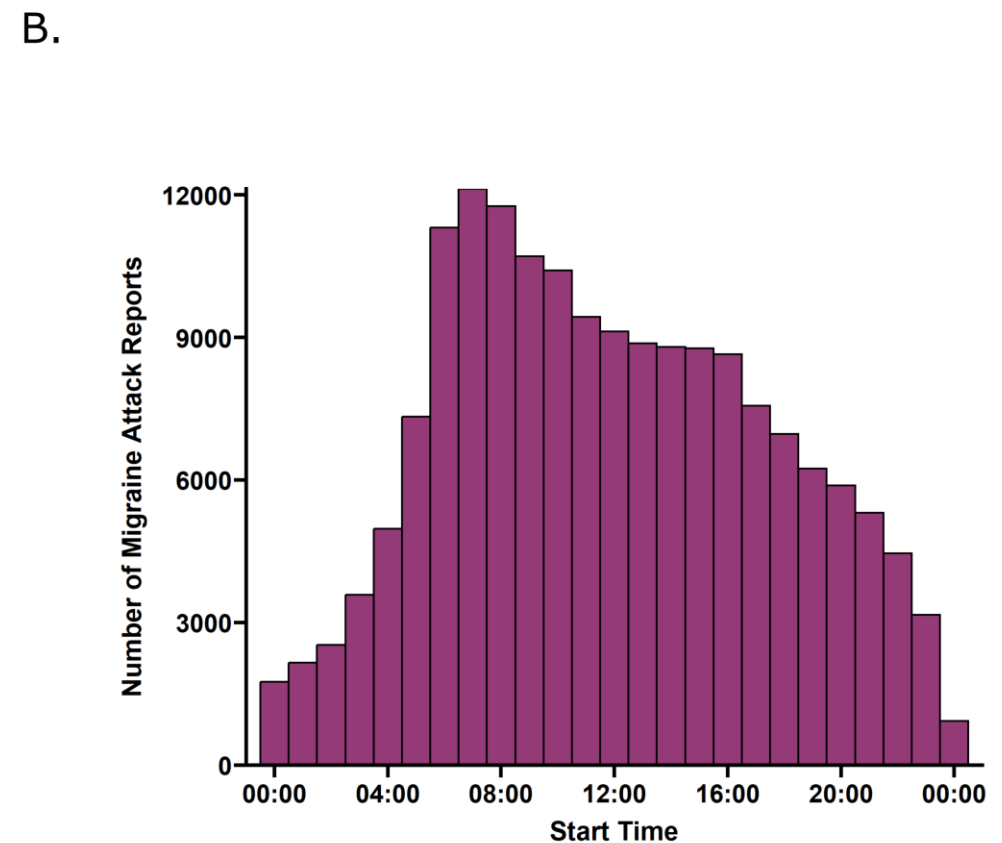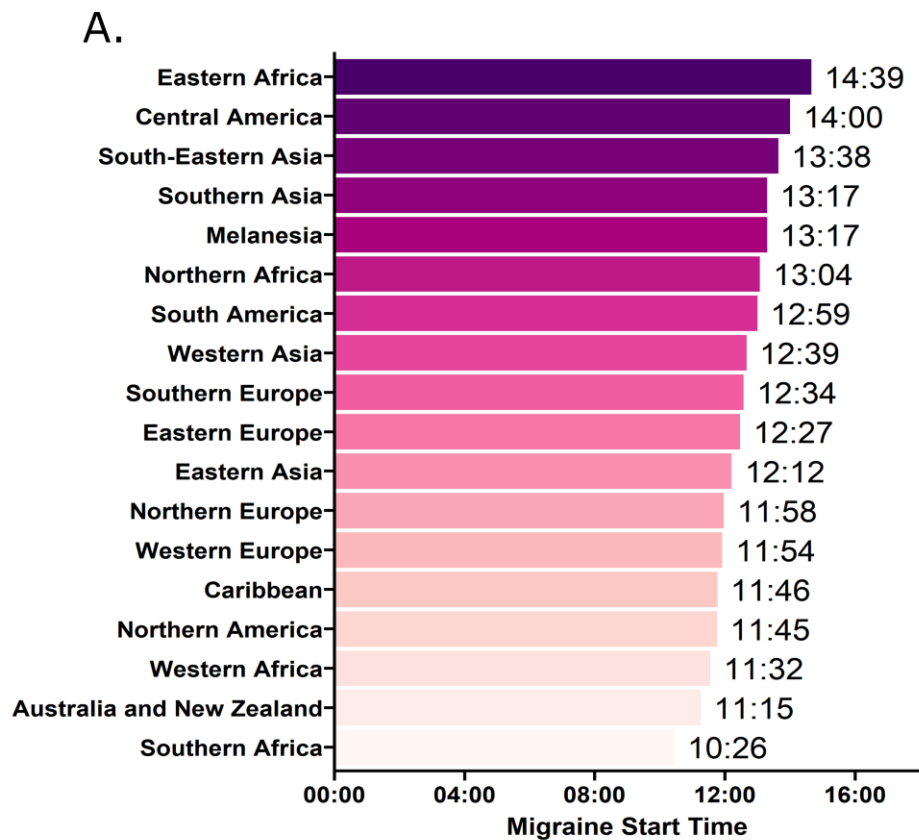
A) Self-reported migraine start times in clock time according to geographical region. Users in Southern Africa had the earliest attack onset of 10:26, whereas users in Eastern Africa had the latest average onset time of 14:39. **B)** The number of migraine attack reports in general over the six-month period and their respective start time. The majority of reported attacks had an onset of around 7:00 or 08:00am.

Table 17: Descriptive statistics for the sleep variables for the overall sample

	Overall sample	Older adults	Young adults	Age unknown	Female	Male	Gender unknown
TST (hrs)	7.45 (1.00)	7.46 (1.04)	7.53 (0.95)	7.43 (1.00)	7.55 (1.00)	7.36 (0.96)	7.34 (1.01)
Sleep Start Time (hrs)	23.55 (1.34)	23.40 (1.42)	23.75 (1.41)	23.55 (1.29)	23.50 (1.38)	23.62 (1.35)	23.60 (1.27)
Sleep End Time (hrs)	31.21 (1.24)	31.10 (1.27)	31.54 (1.23)	31.17 (1.22)	31.28 (1.25)	31.15 (1.31)	31.13 (1.19)
Time in Bed (hrs)	7.66 (1.22)	7.71 (1.28)	7.79 (1.27)	7.62 (1.18)	7.78 (1.25)	7.53 (1.10)	7.54 (1.18)
Time Awake (hrs)	0.21 (0.69)	0.25 (0.76)	0.26 (0.78)	0.19 (0.64)	0.23 (0.75)	0.17 (0.51)	0.19 (0.63)
Sleep Efficiency (%)	0.99 (0.03)	0.98 (0.04)	0.98 (0.04)	0.99 (0.03)	0.99 (0.04)	0.99 (0.03)	0.99 (0.03)
Sleep Interruptions	0.12 (0.18)	0.13 (0.20)	0.13 (0.18)	0.12 (0.18)	0.12 (0.19)	0.10 (0.17)	0.13 (0.17)

Data are represented as mean (SD). Abbreviations: hrs = hours; TST = total sleep time. Sleep start time is from midnight.

Table 18: Descriptive statistics for the migraine variables for the overall sample

	Overall sample	Older adults	Young adults	Age unknown	Female	Male	Gender unknown
Pain intensity (VAS)	5.26 (1.64)	5.34 (1.63)	5.50 (1.55)	5.19 (1.65)	5.42 (1.59)	4.90 (1.77)	5.14 (1.64)
Attack duration (hrs)	18.15 (14.86)	19.46 (15.65)	17.91 (13.75)	17.70 (14.71)	19.16 (15.22)	14.69 (13.07)	17.72 (14.65)
Number of attacks over 6-month period	19.77 (24.17)	21.72 (24.94)	18.14 (21.87)	19.32 (24.22)	20.62 (24.44)	23.41 (27.71)	17.27 (22.21)
Mens. as trigger (%)	0.23 (0.29)	0.18 (0.28)	0.31 (0.30)	0.24 (0.29)	0.27 (0.30)	0.0 (0.02)	0.25 (0.29)
Attacks per month	2.82 (3.45)	3.10 (3.56)	2.59 (3.12)	2.76 (3.46)	2.95 (3.49)	3.34 (3.96)	2.47 (3.17)
Migraine start (hrs)	11.95 (3.31)	11.46 (3.14)	12.96 (3.18)	11.96 (3.35)	5.42 (1.59)	4.89 (1.77)	5.14 (1.64)
Migraine end (hrs)	30.12 (15.10)	30.91 (15.88)	31.07 (14.04)	29.66 (14.95)	19.18 (15.25)	14.77 (13.10)	17.73 (14.66)

Data are represented as mean (SD). Abbreviations: hrs = hours; VAS = visual analogue scale. Migraine start time is from midnight.

4.4.3 Bayesian Modelling

Some users were excluded based on the migraine frequency requirements (those with ≤8 and ≥25 attacks per month), thus in the Bayesian modelling there were 724 users (129 males, 412 females, 183 gender unknown) with an average age of 41.88 years (SD = 11.63) and mean monthly attack occurrence of 9.94. Descriptive statistics for the subset of users within the Bayesian analysis are shown within **19** and **20.** Posterior distributions show the population mean parameter fits across the two models. These distributions tell us the estimate of the possible values of the parameters given the data.

Table 19: Descriptive statistics for the migraine variables for the Bayesian analysis

	18-30	31-40	41-50	51-70	70+	Age Unknown
	n = 38	*n* = 65	*n* = 62	*n* = 95	*n* =4	*n* = 460
Pain intensity (VAS)	5.00 (1.22)	5.37 (1.19)	5.05 (1.43)	5.06 (1.53)	5.84 (1.05)	4.87 (1.58)
Attack duration (hrs)	13.54 (8.99)	14.95 (8.71)	14.02 (8.90)	12.48 (9.78)	9.77 (4.96)	10.31 (6.91)
Number of attacks over 6-month period	68.58 (21.55)	64.11 (22.16)	70.19 (22.32)	68.68 (19.53)	80.25 (35.39)	70.47 (21.90)
Mens. as trigger (%)	0.23 (0.21)	0.24 (0.21)	0.16 (0.22)	0.028 (0.12)	0.00 (0.00)	0.17 (0.21)
Attacks per month	9.80 (3.08)	9.16 (3.17)	10.03 (3.19)	9.81 (2.79)	11.46 (5.06)	10.07 (3.13)
Migraine start (hrs)	13.23 (1.62)	12.49 (1.76)	11.53 (2.22)	10.99 (2.35)	11.11 (2.67)	11.72 (2.35)
Migraine end (hrs)	26.77 (8.20)	27.44 (8.67)	25.55 (9.41)	23.47 (9.84)	20.88 (7.36)	22.03 (7.22)

Data are represented as mean (SD). Abbreviations: hrs = hours; VAS = visual analogue scale. Migraine start time is from midnight.

Table 20: Descriptive statistics for the sleep variables for the Bayesian analysis

	18-30	31-40	41-50	51-70	70+	Age Unknown
	n = 38	*n* = 65	*n* = 62	*n* = 95	*n* = 4	*n* = 460
TST (hrs)	7.69 (0.68)	7.56 (0.90)	7.66 (1.10)	7.52 (1.13)	8.49 (0.77)	7.44 (1.08)
Sleep Start Time (hrs)	23.76 (1.19)	23.56 (1.30)	23.03 (1.65)	23.25 (1.41)	23.21 (0.41)	23.45 (1.47)
Sleep End Time (hrs)	31.81 (0.98)	31.40 (1.06)	31.07 (1.42)	31.22 (1.30)	31.84 (0.96)	31.19 (1.30)
Time in Bed (hrs)	8.05 (1.06)	7.84 (1.14)	8.04 (1.64)	7.96 (1.54)	8.63 (0.67)	7.74 (1.42)
Time Awake (hrs)	0.36 (0.77)	0.28 (0.83)	0.38 (0.99)	0.44 (0.99)	0.14 (0.10)	0.30 (0.98)
Sleep Efficiency (%)	0.98 (0.036)	0.98 (0.047)	0.98 (0.049)	0.97 (0.056)	0.99 (0.012)	0.98 (0.045)
Sleep Interruptions	0.17 (0.22)	0.15 (0.20)	0.13 (0.21)	0.21 (0.32)	0.081 (0.048)	0.13 (0.20)

Data are represented as mean (SD). Abbreviations: hrs = hours; TST = total sleep time. Sleep start time is from midnight.

4.4.4 Sleep Parameters Predicting Migraine

We sought to explore whether sleep variables could predict a next day migraine attack (see **Figure 32**). Deviation from mean sleep duration in hours (M = 0.06, 95% highest density interval (HDI) [0.04 – 0.08]) and number of sleep interruptions yesterday (M = 0.16, 95% HDI [0.11 – 0.21]) were positive predictors of next day migraine attack occurrence, as assessed by the positioning in relation to 0. This means that for every standard deviation from mean sleep, there is a 6.1% increase in odds of having an attack, and for every sleep interruption, there is 17.4% increased odds of an attack occurring. Total hours slept yesterday was not a positive or negative predictor of attack occurrence (M = -0.00, 95% HDI [-0.04 – 0.04]).
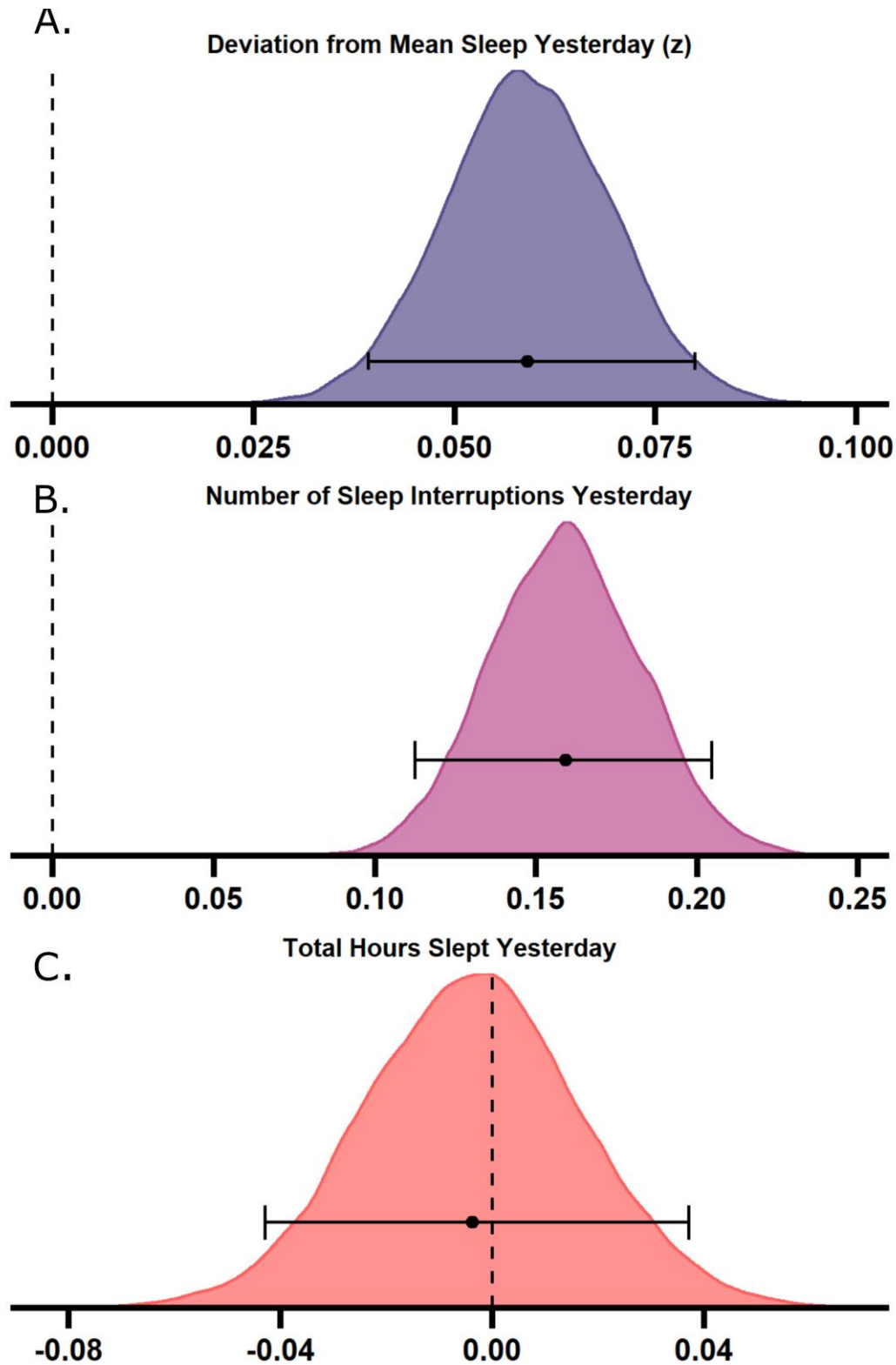
Figure 32: Bayesian posterior distributions for sleep ~ migraine
Larger deviations from typical sleep (**A;** *M* = 0.06, 95% highest density interval (HDI) [0.04 − 0.08])
and a greater number of sleep interruptions (**B;** *M* = 0.16, 95% HDI [0.11 −0.21]) predict occurrence of
a next day migraine attack, but total hours slept does not (**C;** *M* = -0.00, 95% HDI [-0.04 − 0.04]).
Density plots show posterior distributions for the population means of the three tested sleep-related
predictor variables on whether a migraine attack occurred or not. The x-axis represents β-value
estimates of the parameter. Error bars indicate the 95% highest density interval, with the central point
indicating the mean of the posterior distribution.
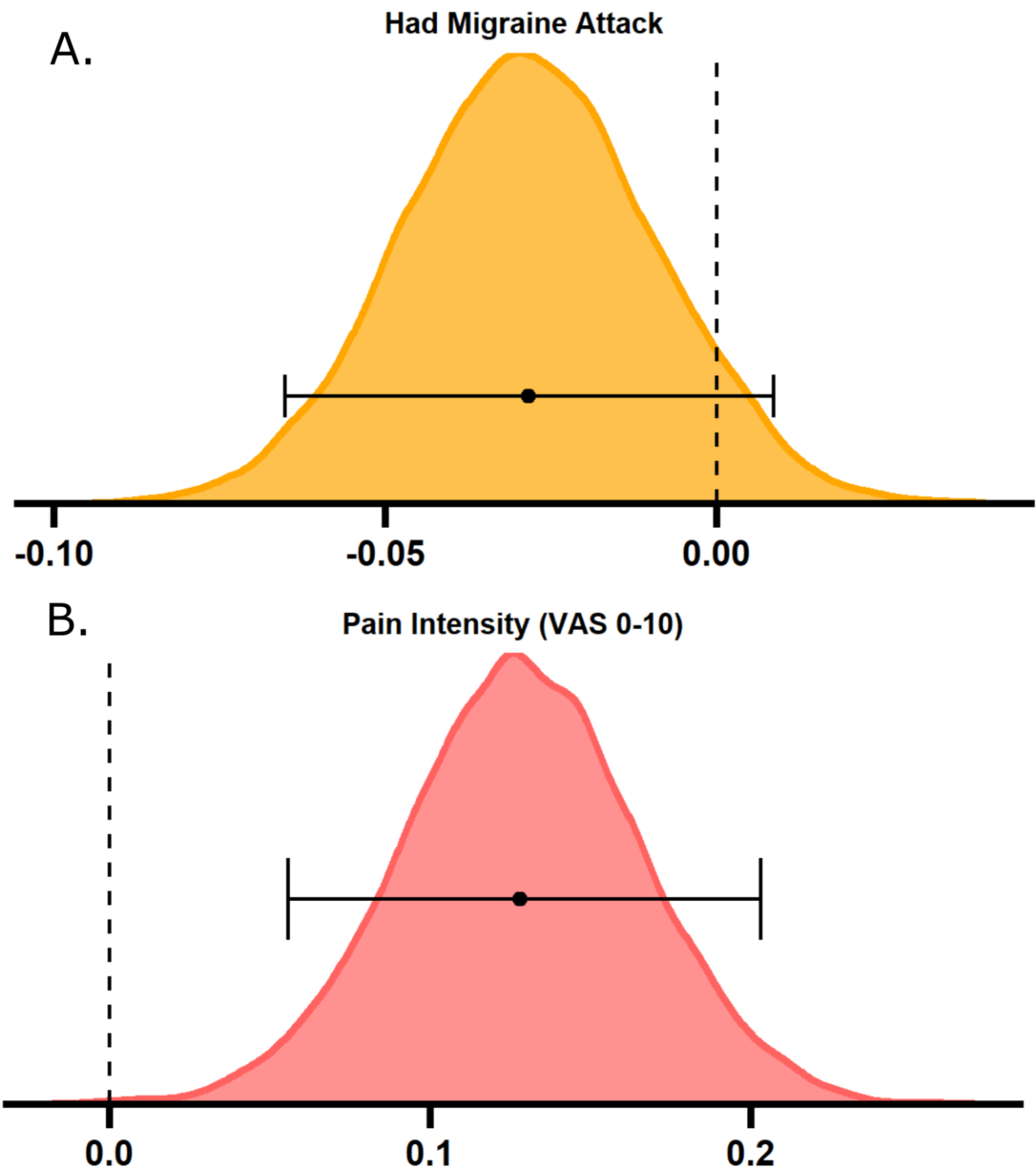
Figure 33: Bayesian posterior distributions for migraine ~ sleep
Having an attack did not predict alterations to sleep (**A;** *M* = -0.03, 95% highest density interval (HDI) [-0.07 - 0.01]), but greater pain intensity (**B;** *M* = 0.13, 95% HDI [0.06 - 0.20]) results in more hours slept the same night. Density plots show posterior distributions for the two tested models of the population means of migraine-related predictor variables on total hours slept. Plots represent the posterior distribution, density estimate. The x-axis represents β-value estimates of the parameter. Error bars indicate the 95% highest density interval. VAS = visual analogue scale.

4.4.5 Migraine Parameters Predicting Sleep

Next, we aimed to investigate the opposite relationship – whether reporting a migraine attack and the self-reported pain associated with that attack could predict alterations in sleep duration (see **Figure 33**). Having a migraine attack was not a predictor of hours slept that evening (M = -0.03, 95% HDI [-0.07 - 0.01]), whereas the pain intensity reported during an attack was a positive predictor (M = 0.13, 95% HDI [0.06 - 0.20]), indicating that for every increase of 1 on the VAS for pain intensity, hours slept increased by 0.13 hours (~8 minutes).

4.5 Discussion

In this chapter we aimed to investigate the relationship between sleep and migraine in a global sample using measures collected from a smartphone application. Using Bayesian regression models, we demonstrated that fragmented sleep, and deviation from typical sleep may predict the occurrence of a migraine attack the next day but sleep duration does not. Thus, we were able to partially support our hypothesis. On the other hand, reporting a more painful migraine attack is predictive of increased sleep duration that same evening, but simply having a migraine attack is not. This contrasts with our hypothesis as we hypothesised that both having an attack and a more painful attack would predict reduced sleep duration. Thus, we were able to disentangle this bidirectional relationship, in that poor sleep increases the risk of next-day migraine occurrence, but having a migraine attack is not sufficient to disrupt sleep. However, the pain intensity associated with migraine attacks alters sleep.

Firstly, we demonstrated that sleep interruptions but not hours slept was associated with greater likelihood of next-day migraine attack occurrence. This aligns with previous work whereby sleep fragmentation but not hours slept was predictive of migraine (Bertisch et al., 2019; Smith et al., 2007), as well as with general literature in which sleep fragmentation predicts, or leads to pain (Iacovides et al., 2017; Rosseland

et al., 2018). Surprisingly, we found that sleep duration was not a predictor of subsequent migraine attack occurrence. Previous studies have found that short sleep duration can predict pain (Edwards et al., 2008) and migraine occurrence (Houle et al., 2012). Moreover, sleeping too long is reported as a trigger for migraine (Inamorato et al., 1993), yet our findings do not support this. This is potentially as previous studies use self-reported diary data and small samples where there is limited variation in habitual sleep duration. The inclusion of patients with migraine from across the world and different cultures in our sample and the automatic detection of sleep could have resulted in the discrepancy here. It is likely that our findings are more reflective of the general population than previous small studies. It is also possible that studies of total sleep duration did not factor in sleep fragmentation (Edwards et al., 2008). Although individuals may have slept for longer durations, it could have been that the sleep during this window was more fragmented – thus leading individuals to seek more time in bed, which was responsible for heightened pain sensitivity.

Unexpectedly, we found that more painful attacks may predict longer subsequent sleep duration, contrary to our hypothesis. Previous literature on pain has shown that pain results in reduced total TST (Edwards et al., 2008; Keilani et al., 2018; Marty et al., 2008; Mulligan et al., 2015; Wylde et al., 2011), or does not affect sleep duration (Lewandowski et al., 2010). The evidence of migraine attacks affecting subsequent sleep duration is lacking. However one prospective questionnaire study in pediatric migraine found that headache intensity was a significant predictor of self-reported sleep disturbances (Heyer et al., 2014). This study did not look at sleep duration *per se*, however. Although we did not include children in the present investigation, this feature may be conserved from children to adults. However, there is little to no evidence reporting an increase in sleep duration due to pain conditions or migraine. Another factor which is important to consider here is that many of these studies used

different methodologies to capture and report sleep variables including smart phone applications, diaries, and actigraphy. It is possible that heterogeneity in sleep variables captured is responsible for the discrepancy in findings. Moreover, this finding could simply reflect recovery or the impact of acute medications on subsequent sleep. For example, if patients are experiencing a painful attack and photophobia they may prefer to lie in a dark room and not use their smartphone. This would be captured by the app as the individual sleeping more when they may simply be reducing sensory input. Future studies could capture the severity of accompanying symptoms including photophobia as well as concomitant acute medication use to address this.

We found using Bayesian regression modelling that deviation from typical sleep duration the night before is associated with greater likelihood of a next day migraine attack occurring. For every deviation from typical hours slept (Z-score), there is a 6.1% increase in odds of having an attack. Whilst previous studies have not explored this directly, these findings align with reports of shift-work and jet-lag being potential risk factors for migraine (Sandoe et al., 2019). Thus, the timing and regulation of sleep rather than the absolute duration of sleep, may be more important for migraine initiation or exacerbation. Indeed, evidence has uncovered potential circadian mechanisms underlying migraine-related phenotypes (Baksa et al., 2019; Brennan et al., 2013; Ong et al., 2018; Solomon, 1992; Strother, 2020), suggesting that alterations in circadian timing could enhance the likelihood of migraine occurrence.

4.5.1 Potential Mechanisms

These results raise further questions on the potential underlying mechanisms of this relationship. We observed that deviation from typical sleep and sleep fragmentation are potential risk factors for migraine attacks. This alludes to a potential role of the hypothalamus in migraine attack initiation. The hypothalamus is involved in homeostatic regulation, modulating relationships between the individual and their

physical environment (Hofman & Swaab, 1992). Migraine is commonly reported to be triggered by changes to the external environment e.g. weather, jet-lag (Kelman, 2007), and many migraine premonitory symptoms (e.g. thirst, yawning, fatigue) pinpoint perturbed homeostatic regulation. The fact that changes in sleep but not total duration may potentially precipitate an attack point to the hypothalamus, as it may be dysfunctional in migraine patients, leading individuals less able to cope with environmental stressors/triggers including sleep disruption.

Indeed, the present results could be explained by dysregulation of the hypothalamic orexinergic system. For example, sleep fragmentation or change in typical sleeping pattern (deviation) may alter the activity of the orexinergic neurons of the hypothalamus (either by their activation or inhibition). In support of this, in animal models, sleep fragmentation causes a reduction in activation of orexinergic neurons (Hunt et al., 2013; Li et al., 2014). This aligns with the proposed role of the orexins in sleep regulation as orexin forms a fundamental part of the flip-flip switch for sleep-wake transitions and stabilizes the waking state (see section 1.5.3). The LH neurons co-express both the pro-nociceptive (OXB) and anti-nociceptive (OXA) orexin peptides (Bartsch et al., 2004). Therefore, we might speculate that sleep fragmentation could result in a reduction of activation of the OXA neurons leading to facilitation of pain and initiation of migraine attacks. This notion is further supported by the prominent fatigue experienced by patients prior to and after attacks.

Alternatively, there could be an underlying dysfunction in the orexinergic system in those with migraine and this could result in sleep fragmentation. Indeed, the prevalence of narcolepsy characterized by a reduction in orexin neurons is higher in those with migraine than the general population (Yang et al., 2017), and migraine is more common in those with narcolepsy (Dahmen et al., 2003) suggesting a potential common underlying pathophysiology related to the orexinergic system. In the present

study we could not predict sleep interruptions from having a migraine attack. This was because for most of the sleep episodes in the dataset, the number of interruptions was very low, and there were difficulties with fitting a predictive model with this small amount of data, thus we focused on sleep duration instead. Future studies using more detailed sleep-tracking methods such as PSG or actigraphy could shed light on this important aspect.

4.5.2 Strengths, Limitations & Future Directions

Despite significant exclusions and reduced sample size from the initial cohort based on frequency requirements, our final sample size remained large in comparison to existing studies. Further strengths are that there was little potential for forgetting as patients reported the start and end times of migraine attacks in real time. Users in the final analysis were based in 38 different countries, across a six-month period. This is in comparison to previous studies which often focus on one region and time point suggesting that the results are generalizable to a wide range of countries with different cultural sleeping habits, sleep practices, and light exposure.

There are some limitations to consider with this study. Most notably, we used estimates of sleep from a smart phone application which determines whether someone is asleep based on whether they pick up their mobile phone. This means we could fail to capture interruptions where the individual did not go on their mobile phone, and subtler changes to sleep architecture. However, users have to approve the estimate of their sleep episode the next morning suggesting that it provides an accurate subjective reflection of their sleep, and smartphone applications have been shown to give accurate estimates of TST (Fino & Mazzetti, 2019). Although, recent research has revealed that migraine patients may overestimate their SOL time (Rantanen et al., 2022). Nonetheless, these results show that despite the cause of the sleep disruption (intrinsic or extrinsic), it can have a considerable impact on migraine likelihood.

Moreover, even with this coarse-grained measure we were able to see that changes in sleep are a significant predictor of migraine attacks, suggesting the utility of datasets collected from smartphone applications for investigating neurological conditions. Future studies could confirm these findings with PSG to determine specific relation to sleep stages and parameters. Indeed, there are commercially-available, low-cost, ambulatory PSG systems (e.g. Dreem headband; https://dreem.com) which have been shown to high validity compared with research-grade PSG (Arnal et al., 2020) suggesting that sleep could be measured accurately over long periods in the home environment.

It is unclear from this study whether changes in sleep are a direct precipitant for attacks or are simply a manifestation of the migraine attack premonitory symptoms, as abnormal tiredness is often reported prior to attacks (Giffin et al., 2003). Nonetheless, changes in sleep detected in this case simply by a smartphone, could be used to identify risk factors of their migraine attack. This could help migraine patients by having a greater understanding of the factors which may be triggering or predisposing them to their attacks. Moreover, we found that more painful attacks may lead to increased sleep duration. This might be explained in that more painful attacks could also be accompanied by increased light sensitivity, and light can exacerbate headaches (Artemenko et al., 2022), and users would not wish to use their phone as much during such attacks.

As touched upon in section 4.3.3, we did not have control over whether users napped, and it was not possible to tell in this analysis if naps were included in the sleep duration measures. However, daytime naps were unlikely to have been included in the analysis as the app sets up automatic detection between times which the user can pre-set (e.g. 22:00-08:00), and therefore would not record outside of these times. Moreover, if naps were included then it is unlikely to be of concern for these findings

as we were interested in sleep duration and fragmentation. Daytime naps usually decrease homeostatic sleep pressure evidenced by an increase in SOL (Campbell et al., 2005) and those who are frequent nappers are shown to have reduced SWS during nocturnal sleep but no difference in TST (McDevitt et al., 2012). Thus, suggesting that napping is unlikely to have confounded the results in this study. Furthermore, as we were interested in understanding sleep in the non-controlled home environment, even if naps were included this would still provide us with an understanding of the relationship.

Furthermore, these studies were collected over a six-month period from June to December encapsulating both summer and winter months across hemispheres. Recent evidence has suggested seasonal changes in REM sleep, with a reduction in the winter months (Seidler et al., 2023) as well as a greater preponderance of migraine attacks during the autumn in Europe (Alstadhaug et al., 2005). Interesting differences in the predictability of migraine attacks using sleep could emerge when comparing seasons and thus future research could explore this, taking into consideration hemispheric differences.

Moreover, another consideration is that although the app collects information covering the ICHD-III criteria (symptoms, duration, location of pain), we did not verify that each individual user had a migraine diagnosis. Other studies using smartphone headache diaries have reported that only 1/3 of headache attacks met the criteria for migraine (Park et al., 2016). Future studies could verify which users could be diagnosed with migraine according to the criteria. Whilst the current study aids our understanding more generally of sleep and headaches, this would allow for more detailed investigations of headache disorders in relation to sleep variables (e.g. migraine with versus without aura). Moreover, we did not analyse any sub-group differences between those with episodic and those with chronic migraine. This is due

to the prevalence of chronic migraine being much lower than episodic in the sample and thus any differences in sleep or prediction would be difficult to interpret.

We defined attacks based on when the user set the start and end time, with the assumption that the user would most likely report an attack start based on throbbing head pain. This means that if there were two attacks in succession these were counted as two separate attacks. However, it is possible that if a patient took acute medication, the pain would be temporarily relieved, and users reported that the attack stopped. However, the pain could resume, and the user could initiate it as a new attack, when it was in fact the same attack, leading to an overestimate of attack frequency. Although, the user was able to amend the start and end times of their attacks, so this is unlikely to be the case. This also highlights the fact that we did not stratify our modelling results by age, yet it is likely that phone usage during the night differed in young versus older adults.

Another consideration is that we did not investigate the presence of sleep disorders, psychiatric co-morbidities, or different chronotypes in this sample, yet it is possible that the relationship between sleep and migraine could differ in those with sleep disorders or between chronotypes. Indeed, recent research has shown that migraine patients tend to be extreme chronotypes versus migraine-free individuals (Sullivan & Martin, 2017; van Oosterhout et al., 2018), and chronotype influences the number and duration of migraine attacks (Viticchi et al., 2019), thus our sample could reflect those with only extreme chronotypes. Furthermore, it could be interesting to compare sleep offset on migraine days versus non-migraine days. In terms of sleep disorders, insomnia commonly manifests as disrupted sleep continuity and insomnia is associated with higher risk of migraine (Alstadhaug et al., 2007; Chu et al., 2021; Kim et al., 2018; Vgontzas & Pavlović, 2018), thus the results herein could be influenced by a high prevalence of insomnia. Moreover, there are psychiatric co-

morbidities with chronic migraine which could be influencing the results herein (Antonaci et al., 2011).

Additionally, it is possible that our results were confounded by medication use. Although this information was collected, we had no control over which medications (acute or preventive) patients were taking and when, and the availability of certain medications could differ across the sample. Indeed, melatonin for headache prevention is available over the counter in the USA which forms a large proportion of the current sample. This being said, melatonin is thought to affect SOL but no other aspects of sleep architecture, thus this may not have affected the results herein (Zhdanova et al., 1996). Future studies could investigate the impact of medication on this relationship, assessing whether some medications are more effective at treating those attacks predicted by poor sleep, or assessing whether treatments delivered at certain times may be more beneficial than others. Moreover, the only pain measure we analyzed was pain intensity. Other measures of pain could also relate to sleep, such as the physical location of pain, and presence and location of allodynia. Alternatively, other measures of sleep could also relate to pain e.g. daytime sleepiness.

4.5.3 Clinical implications

The clinical implications of the current results are that poor sleep could modulate the risk of experiencing a migraine attack. The data suggest that sleep hygiene guidance and sleep interventions may be considered as an inherent part of migraine management (Calhoun & Ford, 2007; Smitherman et al., 2016; Walker et al., 2023) although this hypothesis needs to be confirmed in a dedicated trial that investigates the specific effect of sleeping habits on the course of migraine. As we have shown here, these sleep parameters may be readily detectable by a simple smartphone application meaning minimal input from the patient. This might be aided by the use of sleep diaries and/or wearable sleep monitors (Bianchi, 2018). However, users should be

wary of relying on smartphone measurements of sleep as this may bias subjective sleepiness reports and next-day symptoms (Gavriloff et al., 2018). Rather than focusing on the absolute duration of sleep, clinical care and subsequent research should concentrate on ensuring sleep maintenance and reducing awakenings, to reduce the likelihood of attack occurrence. For example, one potential way of encouraging sleep continuity is through the use of closed-loop auditory stimulation paradigms. For example, 50 ms bursts of pink noise are played to the up-states of slow oscillations during non-rapid-eye-movement sleep which enhances the depth of slow wave sleep and may ensure sleep continuity and maintenance (Stanyer et al., 2022). Moreover, we demonstrated that more painful attacks reduce sleep duration. Therefore focusing on reducing pain associated with attacks through treatments which are unlikely to cause medication overuse headache (Saengjaroentham et al., 2020), rather than focusing on total attack prevention, could lessen the impact on subsequent sleep. As poor sleep is also associated with a greater likelihood of an attack occurring, this then might reduce the likelihood of attack occurrence and chronification. See section 8.2 in the general discussion for a more detailed discussion of potential sleep interventions.

4.5.4 Conclusion

This study aimed to disentangle the relationship between sleep and migraine. Sleep interruptions and deviation from average hours slept are associated with greater likelihood of a migraine attack occurring, whereas overall sleep duration is not. Conversely, simply experiencing a migraine attack does not predict altered sleep duration but having a more intensely painful attack is associated with greater likelihood of increased subsequent sleep duration. Clinicians should ensure sleep hygiene is intrinsic to migraine management and poor sleep may be considered a risk factor for migraine attacks. Longitudinal studies with PSG and more detailed

investigations of headache disorders classified according to ICHD-III criteria are required to confirm these findings, and methods for improving sleep continuity should be explored as a potential migraine intervention.

Chapter 5: Migraine-related Phenotypes Following Sleep-Deprivation

5.1 Introduction

Our prior studies revealed that sleep is significantly altered in migraine patients and that poor sleep may be a risk factor for an ensuing migraine attack. However, what is unclear from these findings is whether poor sleep is merely a symptom of the migraine cycle, including the premonitory phase, or whether it may be involved in the initiation of migraine attacks. To explore this, we used a preclinical mouse model of sleep deprivation allowing direct and controlled investigation of the impact of sleep deprivation on trigeminal pain processing. It is important to note that this is just one aspect of migraine pathophysiology and there are likely higher brain structures involved that may influence attack timing and symptomology which are not explored herein.

Orofacial allodynia is a prominent symptom experienced by up to 70% of patients during migraine attacks (Guy et al., 2009; Lipton et al., 2008), in which they experience hypersensitivity to non-painful stimuli such as a brush or touch (Goadsby, 2005; see section 1.1.5). Allodynia is predominantly cephalic in nature, is a key feature of attacks, and is considered a marker of migraine chronification (Benatto et al., 2017). Allodynia can be readily assessed in both preclinical and clinical studies as a readout of migraine-related phenotypes. For example, in humans, attack provocation studies using CGRP and nitroglycerin (NTG) have been shown to elicit mechanical orofacial allodynia (De Logu et al., 2019) and a similar approach can be used in preclinical models to explore translational outcomes (Moye & Pradhan, 2017; Pradhan et al., 2014)

Allodynia can also be evaluated in the thermal domain (Mínguez-Olaondo et al., 2022), and in humans thermal pain thresholds are typically quantified using quantitative sensory testing (Yarnitsky, 1997). In animal models, thermal allodynia is commonly quantified using the hot plate or Hargreaves's test (Hargreaves et al., 1988;

Tjølsen et al., 1991), or cold thresholds are assessed using the acetone test (Vuralli et al., 2019). These are assumed to be valid readouts of allodynia, as migraine-specific treatments such as sumatriptan have been shown to alleviate thermal allodynia in mice (Bates et al., 2009). Notably, these assays are usually localised to the hind paw in animals and the arm in humans and therefore may have limited utility in investigating head-specific pain.

In animal models, mechanical periorbital allodynia is commonly assessed by the von Frey assay – applying calibrated, graded filaments to the cephalic (orofacial) or extracephalic (hind paw) regions and determining evoked response thresholds (Chaplan et al., 1994; Pradhan et al., 2014, 2014). Importantly, studies have shown that NTG (both acute and chronic administration) elicits hyperalgesia in animals using this model, and that this can be reversed using migraine preventives (Tipton et al., 2016). This highlights its use as a potential screening tool for novel migraine therapies and as a valid readout of migraine-related phenotypes (Harriott, Strother, et al., 2019).

In relation to sleep, clinical studies have revealed that migraine patients who experience mechanical allodynia exhibit a greater frequency of sleep disruptions than patients which do not (Lovati et al., 2010), and allodynia is negatively correlated with subjective sleep duration (de Tommaso et al., 2014) highlighting a relationship between mechanical allodynia and sleep. Using direct manipulations to sleep, animal studies have demonstrated that brief REM sleep deprivation induces orofacial mechanical allodynia (Kim et al., 2019). However, this study did not investigate the impact of acute total sleep deprivation (deprivation of both REM and NREM sleep) on orofacial allodynia which more closely mirrors typical sleep deprivation in humans. Moreover, this study conducted REM sleep deprivation using the platform over water method (Jouvet et al., 1964) and this has been shown to also reduce NREM sleep (Arthaud et al., 2015) and lead to elevated physiological stress levels (Coenen & Van

Luijtelaar, 1985), suggesting that it may not be the most appropriate method of sleep deprivation.

Alternatively, studies have used the gentle handling method (GHM) of total sleep deprivation (see section 2.4; Franken et al., 1991; Tobler & Borbély, 1990) in which mice are left relatively undisturbed until they show signs of the forbidden state or sleep stage, measured either behaviourally or using EEG. The animal is subsequently kept awake by introducing novel objects into the cage, changing the bedding, or by providing acoustic or tactile stimuli, only when required. Utilising this method, chronic sleep deprivation but not chronic sleep fragmentation has been shown to elicit extracephalic allodynia (Alexandre et al., 2017; Sutton & Opp, 2014). This is at odds with the findings in **Chapter 4** in human patients in which sleep fragmentation but not hours slept were predictive of a next day migraine attack, as well as previous literature (Bertisch et al., 2019; Smith et al., 2007). Mixed findings have also been reported for thermal allodynia in that acute sleep deprivation does (Alexandre et al., 2017) and does not elicit (Yu et al., 2023) thermal extracephalic allodynia. Alternatively, prior sleep deprivation may render an individual more susceptible to migraine. For example, a recent study in mice found that six hours of acute sleep deprivation did not elicit mechanical allodynia. However, six hours of sleep deprivation prolonged the extracephalic allodynia induced by NTG (Yu et al., 2023).

As well as developing a greater understanding of the impact of sleep deprivation on cephalic and extracephalic allodynia, in this chapter we aimed to explore potential underlying mechanisms and interventions. Previous studies have demonstrated a significant increase in neural activation in the vlPAG, and the posterior and dorso-medial hypothalamus measured using c-Fos IHC following REM sleep deprivation. This suggests a potential alteration in descending modulation of nociception following sleep deprivation (Kim et al., 2019), as both the PAG and hypothalamus have been

implicated in descending pain modulation through connections with the TCC (Tomim et al., 2016). Notably however, this study did not investigate the impact of total sleep deprivation on orofacial allodynia, therefore, it is unclear how total sleep deprivation affects orofacial allodynia and neural activation in pain-processing pathways.

Another plausible mechanism of migraine attack initiation is that sleep-disruption-dependent modulation of trigeminal sensory processing could alter CGRP signalling. CGRP is a neuropeptide found extensively within the CNS and peripheral nervous system (van Rossum et al., 1997; Warfvinge & Edvinsson, 2019). Increased central and peripheral CGRP levels have been demonstrated in patients with episodic and chronic migraine (Bellamy et al., 2006; Cernuda-Morollón et al., 2013; Goadsby et al., 1990; Goadsby & Edvinsson, 1993), and CGRP infusion has been shown to trigger migraine-like headaches (Hansen et al., 2010). Many effective migraine therapies work by blocking CGRP release directly or indirectly highlighting an important role of CGRP in migraine (de Vries et al., 2020).

Interestingly, CGRP has been reported to mediate sleep maintenance and arousal via homeostatic sleep mechanisms (Kaur et al., 2017; Kunst et al., 2014). Furthermore, plasma CGRP levels exhibit clear circadian variation (Trasforini et al., 1991; Wimalawansa, 1991), being maximal during the night, with the majority of migraine attacks occurring during the early hours of the morning (see **Chapter 4; Figure 31**), suggesting a potential link between CGRP and sleep-wake regulation. CGRP monoclonal antibody treatment has been shown to potentially modulate chronotype with a shift from morning to intermediate type in chronic migraine patients, as well as lead to improvements in sleep quality (Pellitteri et al., 2022). This suggests either an influence of CGRP on circadian rhythms or treatment of headache symptoms being associated with alterations in sleep and sleep timing (Pilati et al., 2023). Given the findings in the previous chapter on sleep disruption predicting attacks, the role of

CGRP in migraine attacks and sleep regulation, we postulate that sleep deprivation may alter CGRP-dependent mechanisms in trigeminal sensory afferents.

5.1.1 Aims and Hypotheses

In this chapter we aimed to explore whether acute and chronic total sleep deprivation in mice induces orofacial mechanical and extracephalic thermal allodynia. We also aimed to investigate whether sleep deprivation stimulates neural activity in key brain regions involved in nociceptive transmission and migraine; specifically, the LH and PAG. Furthermore, we aimed to establish whether sleep deprivation may mediate migraine-related phenotypes via CGRP signalling by assessing CGRP expression in regions involved in trigeminal pain processing following acute sleep deprivation.

We hypothesise that: *i)* acute sleep deprivation, as well as both consecutive and intermittent chronic sleep deprivation will elicit orofacial allodynia as measured by reduced mechanical withdrawal thresholds in line with previous literature of reduced thresholds in the hind paw (Alexandre et al., 2017); *ii)* this will be accompanied by an increase in neural activation in the LH and PAG as measured by c-Fos IHC (Kim et al., 2019); *iii)* acute sleep deprivation will induce thermal allodynia as measured by reduced latency on the hotplate test (Alexandre et al., 2017); *iv)* there will be a significant increase in CGRP expression in the TCC following acute sleep deprivation.

5.2 Methods

5.2.1 Validating the Sleep Deprivation Method

There are various methods to elicit sleep deprivation in mice (Gulyani et al., 2000; Nor et al., 2021), including automated and mechanical methods (Amici et al., 2000; Bergmann et al., 1989; Gervais & Pager, 1979; Leenaars et al., 2011; Nunes & Tufik, 1994; Singh & Nath Mallick, 1996; Van Hulzen & Coenen, 1980; Van Twyver et

al., 1966), however these are subject to ethical and methodological criticisms. For example, many methods selectively deprive mice of REM sleep using platforms over water which induces stress from hydrophobia and social isolation (Suchecki & Tufik, 2011), and efficacy may vary widely dependent on the weight and size of the animal (Vogel, 1975). The GHM on the other hand, has been shown to completely abolish REM sleep and reduce NREM sleep to 92% and 98% of baseline levels respectively (Alexandre et al., 2017; Franken et al., 1991). This method does not lead to significant increases in physiological stress (Kalinchuk et al., 2010), and is relatively easy to administer.

Therefore, we aimed to recapitulate previous findings in that the GHM significantly reduces sleep and is less likely to induce physiological stress. This is important as behavioural testing can vary widely depending on a variety of experimental factors and across laboratories (Freymann et al., 2017; Sultana et al., 2019). Moreover, stress is a key confound as stress alone elicits mechanical and thermal allodynia in animal models (Bardin et al., 2009; Ito et al., 2023) and humans (Crettaz et al., 2013).

To this end, we conducted a validation study comparing the GHM, with direct handling, and no intervention (see **Figure 34**). Direct handling was chosen as a positive control due to handling alone increasing physiological stress levels in mice (Le Cudennec et al., 2002; Novak et al., 2022). Specifically, 12 mice were randomly allocated to one of three groups: direct handling (n = 4), GHM (n = 4) and no intervention (n = 4). To assess any changes in physiological stress, baseline blood samples were taken via tail vein sampling as described in section 2.7.1 at the same circadian time as the end of sleep deprivation (ZT6) for measurement of plasma corticosterone levels. Animals had not been disturbed by the experimenters or had their cage material changed for one week prior to the study, as we previously observed

significant circadian phase shifts following cage changes (unpublished observation), which could affect corticosterone levels (Atkinson & Waddell, 1997). On day two at the start of the light cycle (ZT0) the animals in the GHM were sleep deprived as described in section 2.4. In the direct handling group, when mice attempted sleep, they were lifted from the cage by the experimenter and handled for two minutes. At ZT6 final blood samples were taken via tail vein sampling. All mice were sampled in the same session.
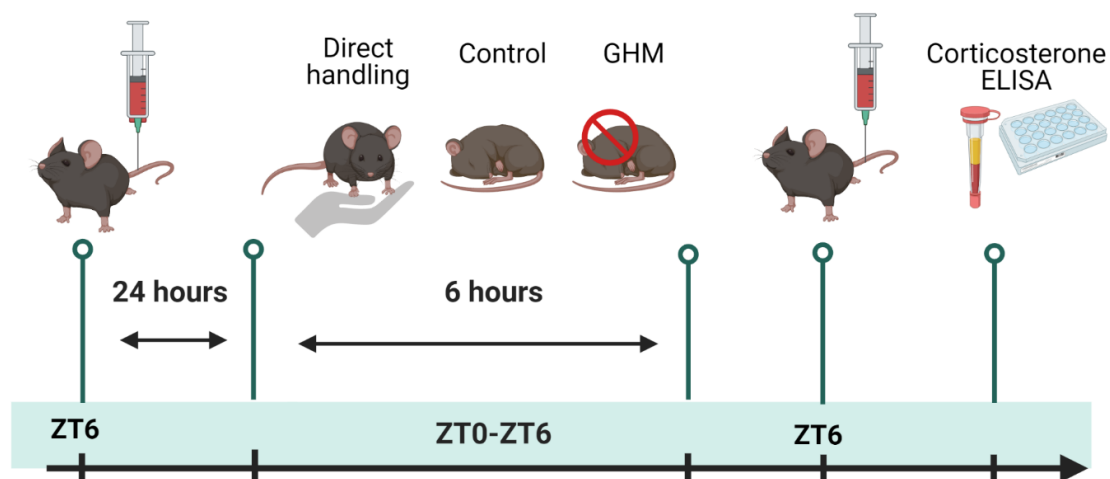
Figure 34: Timeline demonstrating the analysis of corticosterone levels
Tail vein blood samples were taken at ZT6 24 hours prior to the end of sleep deprivation. Mice then received either six hours of acute sleep deprivation using the gentle handling method (GHM), direct handling for six hours or were left to sleep as usual from ZT0-ZT6. At ZT6, tail vein blood samples were taken away. Samples were centrifuged to extract plasma and were analysed using an ELISA kit for corticosterone. Abbreviations = GHM = gentle handling method; ZT = Zeitgeber time.

We additionally sought to confirm previous findings that the GHM significantly reduces sleep by ~90% of baseline levels (Franken et al., 1991). To this end, 11 mice in a separate cohort were implanted with EEG and EMG electrodes as described in section 2.3.2 and data were acquired over a 24-hour baseline period (see **Figure 35**). One week after baseline recording, mice were returned to the recording chamber at ZT9 to acclimatise. Mice were then sleep deprived using the GHM as previously described in section 2.4 for six hours from "lights on" (ZT0–ZT6). Mice were then

allowed to sleep as usual for three hours until ZT9 during which EEG and EMG recording continued.
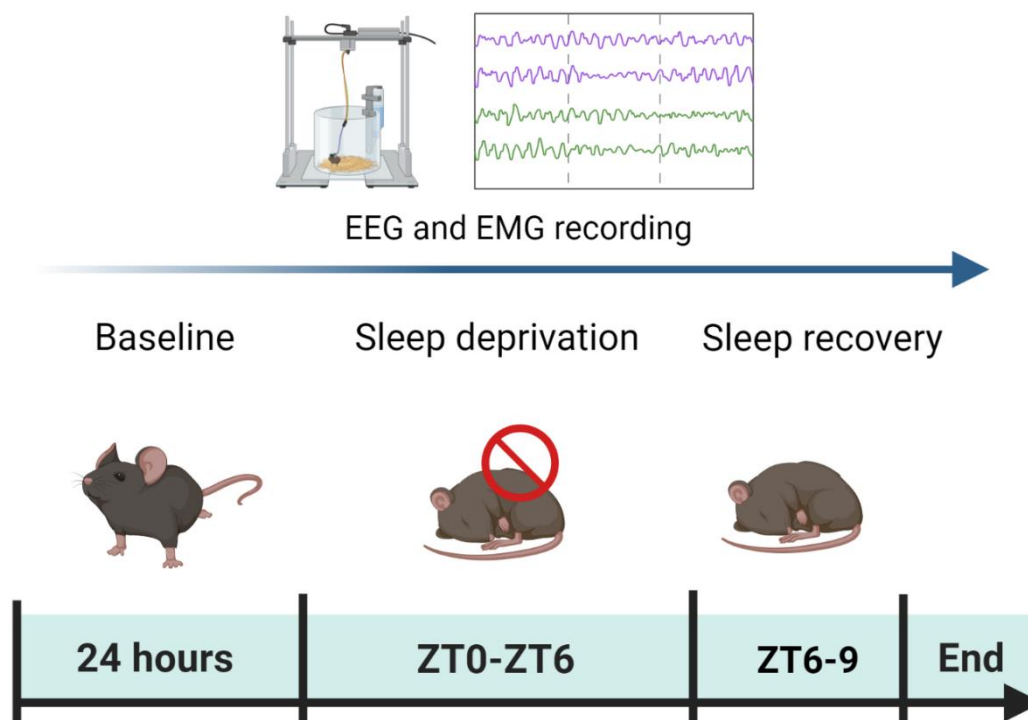
Figure 35: Timeline of the sleep deprivation EEG validation study
EEG and EMG were recorded in wild-type mice for 24 hours to provide a baseline measurement. Following a 24-hour rest period, mice were then sleep deprived for six hours from ZT0-ZT6 using the gentle handling method. Mice were allowed to sleep as usual for three hours from ZT6-ZT9. Recording continued for the entire period. Abbreviations: ZT = Zeitgeber time.

5.2.2 Plasma Corticosterone

Levels of plasma corticosterone were measured using an ELISA kit as described in section 2.7.4. A 3x2 mixed analysis of variance (ANOVA) was conducted with session (pre/post) as the within-subjects independent variable (IV), condition (direct handling, GHM, control) as the between-subjects independent variable, and corticosterone (ng/ml) as the dependent variable.

5.2.3 EEG

EEG data were manually scored as described in section 2.3.2. For statistical analysis, the minutes spent in each sleep stage (NREM, REM, wake), mean sleep stage length (in minutes), total sleep stage shifts, arousals (>10s wake after NREM/REM

sleep), NREM and REM sleep latency (in minutes), the number of sleep stage episodes and mean sleep stage episode duration were computed for each mouse for the baseline and sleep deprivation recordings for six hours from ZT0-ZT6. The percentage of total sleep (both NREM and REM) over the recording period were also computed for both sessions. REM and NREM were combined to give an overall measure of sleep and visualised in 1-hour windows. For visualisation purposes, cumulative probability distributions of the duration of stage bouts (in minutes) were computed for each sleep stage. For all sleep variables, paired t-tests with the Bonferroni correction for multiple comparisons were computed between baseline and sleep deprivation conditions.

5.2.4 Thermal Sensitivity

Thermal sensitivity was assessed using the hot plate test as described in section 2.6.4 at baseline (ZT6; see **Figure 36**). 24 hours later, mice were randomly allocated to receive either six hours of acute sleep deprivation using the GHM (n = 12) or no sleep deprivation (n = 12) from ZT0. Afterwards, at ZT6, thermal latencies were assessed. Data were assessed by mixed ANOVA with latency to display a nociceptive response (time until the animal flicked either hind paw) in seconds as the dependent variable.
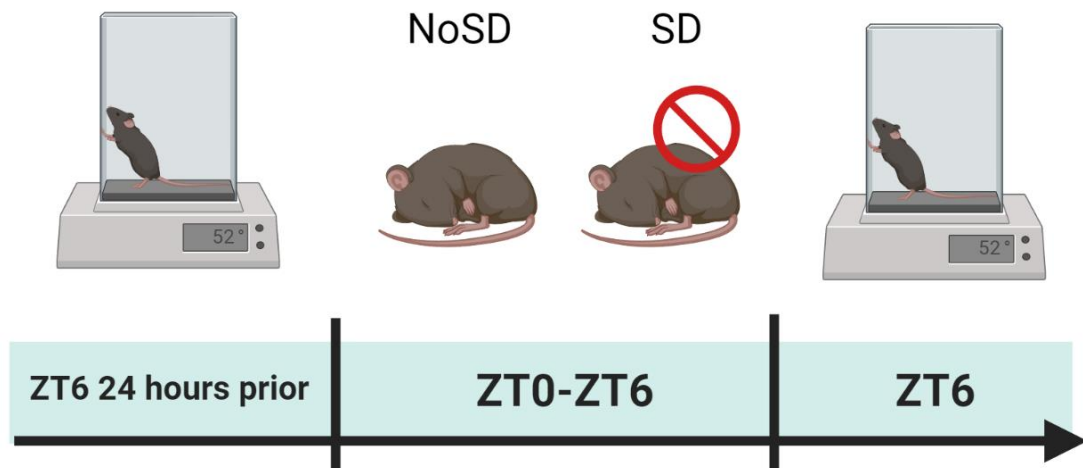
Figure 36: Timeline of thermal sensitivity following acute sleep deprivation
Baseline hotplate latencies (thermal extracephalic sensitivity) were established in all mice at ZT6 24 hours prior to the end of sleep deprivation. Mice were then counterbalanced to received acute sleep deprivation (n = 12) or sleep as usual (n = 12) from ZT0-ZT6. Hotplate latencies were then measured immediately after at ZT6.

5.2.5 Mechanical Sensitivity

Intermittent sleep deprivation

24 mice (12 male, 12 female) were used in this experiment. After measurement of baseline mechanical thresholds, mice were randomly allocated to one of three conditions: six hours of sleep deprivation (6hrSD; n = 8), three hours of sleep deprivation (3hrSD; n = 8), or no sleep deprivation (NoSD; n = 8). We chose to conduct three hours as well as six hours as it was not known whether three was sufficient to induce allodynia, and previous studies had only investigated six hours. Sleep deprivation was conducted using the GHM as described in the methods section 2.4 on three days separated by 24 hours (see **Figure 37**). For the six-hour sleep deprivation condition this was conducted from ZT0–ZT6 and for the three-hour condition this was conducted from ZT0–ZT3. We chose to only conduct three days of sleep deprivation due to ethical considerations and the finding that physiological stress has been shown to increase on day six of the GHM (Longordo et al., 2011).
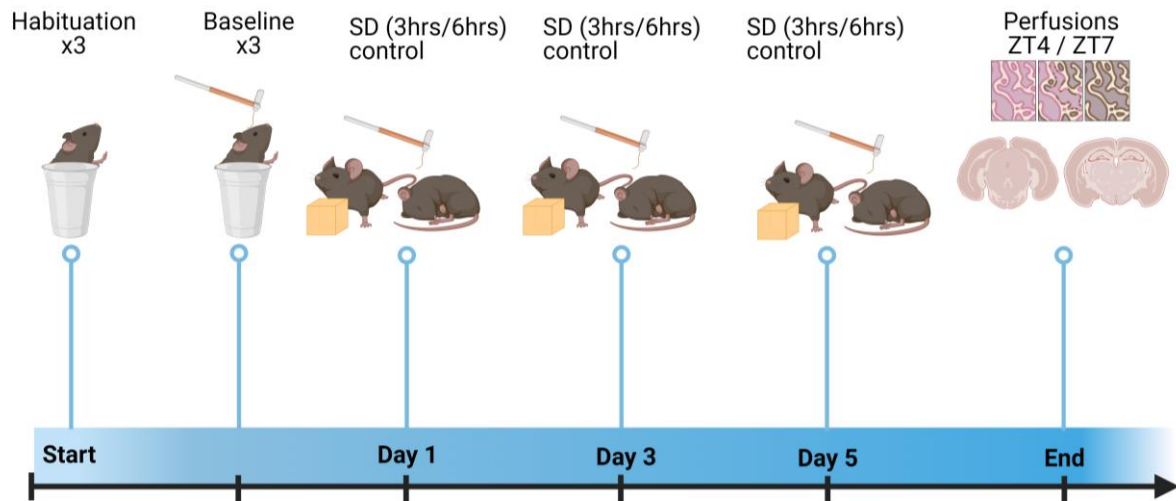
Figure 37: Experimental timeline for intermittent sleep deprivation protocol
Mice were habituated three times to the von Frey apparatus and three baseline measurements were subsequently taken which were separated by three days to provide an average baseline measurement. Mice were then sleep deprived for either six ($n = 8$), or three hours ($n = 8$), or left to sleep as usual ($n = 8$). Periorbital mechanical withdrawal thresholds were assessed following each sleep deprivation instance. On day five, mice were terminated at ZT7 (6hrSD) or ZT4 (3hrSD) using perfusion fixation which was assessed for c-Fos immunohistochemistry.

Periorbital thresholds were measured as described in the methods section 2.6.1. To account for circadian variation in thresholds, four control mice were tested at ZT3 and the other four at ZT6 to correspond to the timing of testing in the two experimental conditions. However, mechanical withdrawal thresholds in the periorbital region have been shown to be consistent between these times therefore we did not anticipate any significant circadian influence on thresholds (Strother, 2020).

At the end of the sleep deprivation period (ZT6 or ZT3), mice were placed in the von Frey apparatus for 30 minutes to habituate before testing. During this time, mice were monitored carefully to ensure they did not sleep. If any mouse in the sleep deprivation conditions showed signs of sleep, acoustic stimulation was provided by gently tapping the side of the box. The procedure was repeated on two more days, with a day in-between testing. At the end of sensory testing on day five for the 3hrSD, 6hrSD (ZT4 and ZT7 respectively) and non-sleep-deprived control group the mice were euthanised as described in section 2.7.2. Data were statistically assessed by mixed

ANOVA, and post-hoc tests were conducted using pairwise Student's *t*-tests with the Bonferroni correction for multiple comparisons applied.

c-Fos expression

A standard IHC approach was used to establish c-Fos activity in the LH, ventrolateral PAG (vlPAG), and dorsolateral PAG (dlPAG) as described in section 2.7.3. Whilst the PAG has no clear cytoarchitectural boundaries, distinct behavioural effects, and afferent and efferent connections have resulted in the PAG being divided into distinct areas around the aqueduct (Keay & Bandler, 1993). In the current experiment we investigated c-Fos expression in the dlPAG and vlPAG subdivisions based on previous experiments (Kim et al., 2019). The mean number of c-Fos+ cells per coronal section in each region of interest (ROI) was calculated. Differences in the number of c-Fos+ cells were computed using one-way ANOVAs for each ROI (dlPAG, vlPAG, LH). Where appropriate, post-hoc tests were conducted using unpaired Student's *t*-tests with the Bonferroni correction for multiple comparisons.

Consecutive sleep deprivation

24 C57bl/6J mice (12 male, 12 female) were used in this experiment. The protocol was identical to above. However, sleep deprivation was conducted on three consecutive, rather than intermittent days (see **Figure 38**). There was no significant difference in baseline thresholds between sleep deprived and non-sleep-deprived mice (0.60 ± 0.40 vs 0.56 ± 0.27; $t_{19.2}$ = -0.32, p = 0.765). Furthermore, at the end of the third threshold testing (ZT7), mice were afforded a three-hour sleep opportunity until ZT10. Note that this is one hour later than the sleep recovery period for the EEG validation (ZT6-9), due to the time taken to habituate and complete the sensory testing. Mice were habituated to the von Frey apparatus for 30 minutes again before the final threshold assessment. Differences in thresholds were assessed using a mixed

ANOVA. Where appropriate, post-hoc tests were conducted using pairwise Student's *t*-tests with the Bonferroni correction for multiple comparisons.
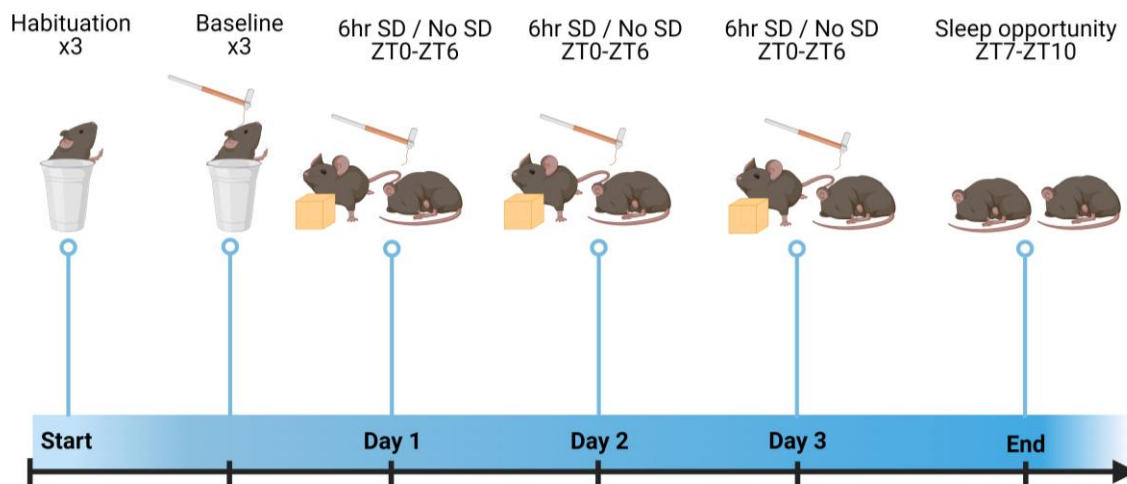
Figure 38: Experimental timeline for consecutive sleep deprivation
Mice were firstly habituated to the von Frey apparatus and then three baseline measurements were taken which were separated by three days. Mice were then either sleep deprived for six hours (*n* = 12) or left to sleep as usual (*n* = 12), on three consecutive days. Periorbital mechanical withdrawal thresholds were assessed following each sleep deprivation instance. On day three, all mice were given the opportunity to sleep for three hours until ZT10 where periorbital thresholds were assessed again.

5.2.6 CGRP Expression

We then explored CGRP expression in the TCC following acute sleep deprivation. 22 mice were included in this experiment (11 male, 11 female). Mice were administered either 10 mg/kg NTG (*n* = 8), saline (*n* = 8) or six hours of sleep deprivation (*n* = 6) as described above. NTG administration was chosen as a positive control as NTG triggers attacks in human migraine patients (Thomsen et al., 1994) and leads to elevated CGRP levels (Greco et al., 2018; Wei et al., 1992). Perfusions were conducted two hours post injection as previous studies have demonstrated a peak in NTG-evoked hyperalgesia two hours post administration (Pradhan et al., 2014). Mice were immediately perfused as described in section 2.7.2 and spinal cords were extracted. All perfusions were conducted at the same time (ZT6) to control for circadian variation in CGRP levels (Wimalawansa, 1991). Expression of CGRP immunopositive fibres in the TCC was

determined using fluorescent IHC as described in section 2.7.1. Data were assessed by one-way ANOVA and unpaired *t*-tests with the Bonferroni correction applied.

For all the below experiments, data are reported as mean ± standard deviation, and statistical significance was set at $\alpha = 0.050$ unless otherwise stated.

5.3 Results

5.3.1 The gentle handling method does not increase physiological stress

To assess whether the GHM impacts physiological stress responses we measured plasma corticosterone levels in tail vein blood samples following direct handling, GHM, or no sleep deprivation. Two blood samples had to be discarded due to inadequate volume (<30 µl) collected during sampling (final $n = 10$; no sleep deprivation = 3; direct handling = 3; GHM = 4).

There was a significant difference in plasma corticosterone levels between the different conditions ($F(2, 6) = 16.70$, $p = 0.004$, $\eta^2_G = 0.49$). However, corticosterone did not differ across sessions (pre-post; $F(1,6) = 0.44$, $p = 0.530$, $\eta^2_G = 0.06$), and there was no significant interaction ($F(2,6) = 0.38$, $p = 0.766$, $\eta^2_G = 0.07$; **Figure 39**). Direct handling significantly increased plasma corticosterone levels compared to no sleep deprivation (75.4 ± 32.7 vs 35.2 ± 6.35; $t_{2.20} = 3.11$, $p = 0.029$). No other comparisons were significant, in that corticosterone levels were not significantly higher in GHM compared to control mice or compared to their respective baseline.

5.3.2 The gentle handling method decreases sleep

To validate the sleep deprivation method, we conducted EEG and EMG recordings during acute sleep deprivation and sleep recovery. One mouse was excluded after baseline recording due to the head-mounted connector becoming loose, thus their data was not included in the final analysis (final $n = 10$). Detailed descriptive and inferential statistics for the sleep data are shown in **Table 21**.

Sleep deprivation significantly decreased the time spent in NREM (t_9 = 38.0; p < 0.001), REM sleep (t_9 = 13.2; p < 0.001), and increased the time spent awake (t_9 = -38.7; p < 0.001; **Figure 40A**) and decreased the number of arousals (t_9 = -1.34; p < 0.001; **Figure 41A**) compared to the baseline session. REM sleep latency (t_9 = -1.34 = 5.82; p = 0.013; **Figure 40E**) but not NREM sleep latency was significantly longer during the sleep deprivation period (t_9 = 4.67; p = 0.190; **Figure 40D**). Mice had significantly less stage shifts (NREM, REM and wake) during the sleep deprivation period compared to baseline (t_9 = 7.13; p < 0.001; **Figure 41B**), and the mean stage length was significantly increased during sleep deprivation for wake (t_9 = -7.86; p < 0.001), but not for REM (t_9 = -1.83; p = 0.064) and NREM sleep (t_9 = -1.22; p = 0.287; **Figure 40B**).

For the sleep recovery period, there was a significant difference in power spectral density (PSD) in the delta range between baseline and sleep deprivation conditions for the frontal (t_9 = -3.32, p = 0.009) but not the central (t_9 = -1.86, p = 0.096) EEG channel, suggesting a significant sleep rebound (**Figure 42B-C**).

Table 21: Sleep architecture before and during acute sleep deprivation

Variable	Baseline	Sleep Deprivation	t_9	p-value
Time in sleep stage (minutes)				
NREM	197 (10.9)	26.5 (19.8)	38.0	< 0.001
REM	24.6 (5.02)	1.12 (1.78)	13.2	< 0.001
Wake	139 (8.45)	331 (20.4)	-38.7	< 0.001
Sleep stage length (minutes)				
NREM	2.88 (0.53)	3.09 (0.34)	-1.22	0.254
REM	1.10 (0.16)	1.32 (0.33)	-1.83	0.101
Wake	2.00 (0.56)	8.13 (2.37)	-7.86	< 0.001
Arousals	104 (22.0)	48.9 (11.9)	-1.34	< 0.001
Stage shifts	244 (47.1)	114 (22.1)	7.13	< 0.001
Sleep latency (minutes)				
NREM	44.4 (22.9)	74.8 (70.7)	5.82	0.190
REM	64.0 (20.8)	233 (172)	4.67	0.013

Data are represented as mean (SD). Significance computed using paired Student's t-test, where $\alpha = 0.050$. n = 10. Abbreviations: NREM = non-rapid-eye-movement sleep, REM = rapid-eye-movement sleep.
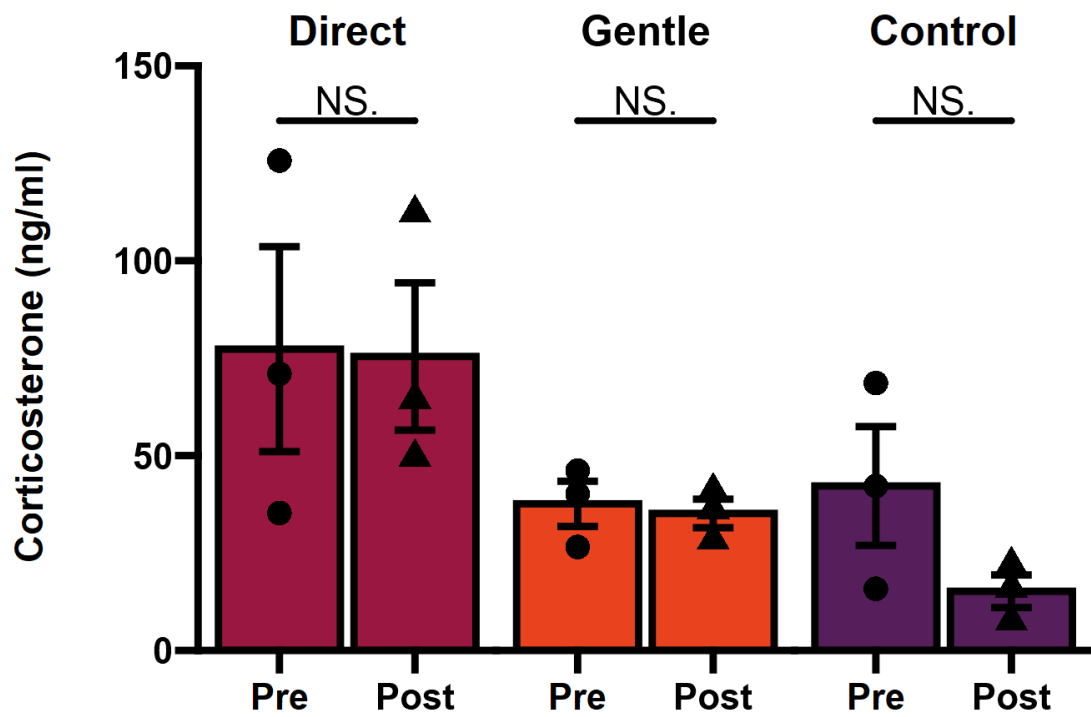
Figure 39: Plasma corticosterone levels following sleep deprivation

Plasma corticosterone levels at baseline (pre), and post intervention in wild-type mice were not significantly different between sleep deprived mice using the gentle handling method (gentle, $n = 4$), directly handled (direct, $n = 3$), or not sleep deprived (control, $n = 3$) for six hours. Data are represented as mean ± SEM. Shapes overlaid on bars represent data from each individual animal. Comparisons were computed using paired Student's t-tests. NS. = not significant at $p < 0.050$.
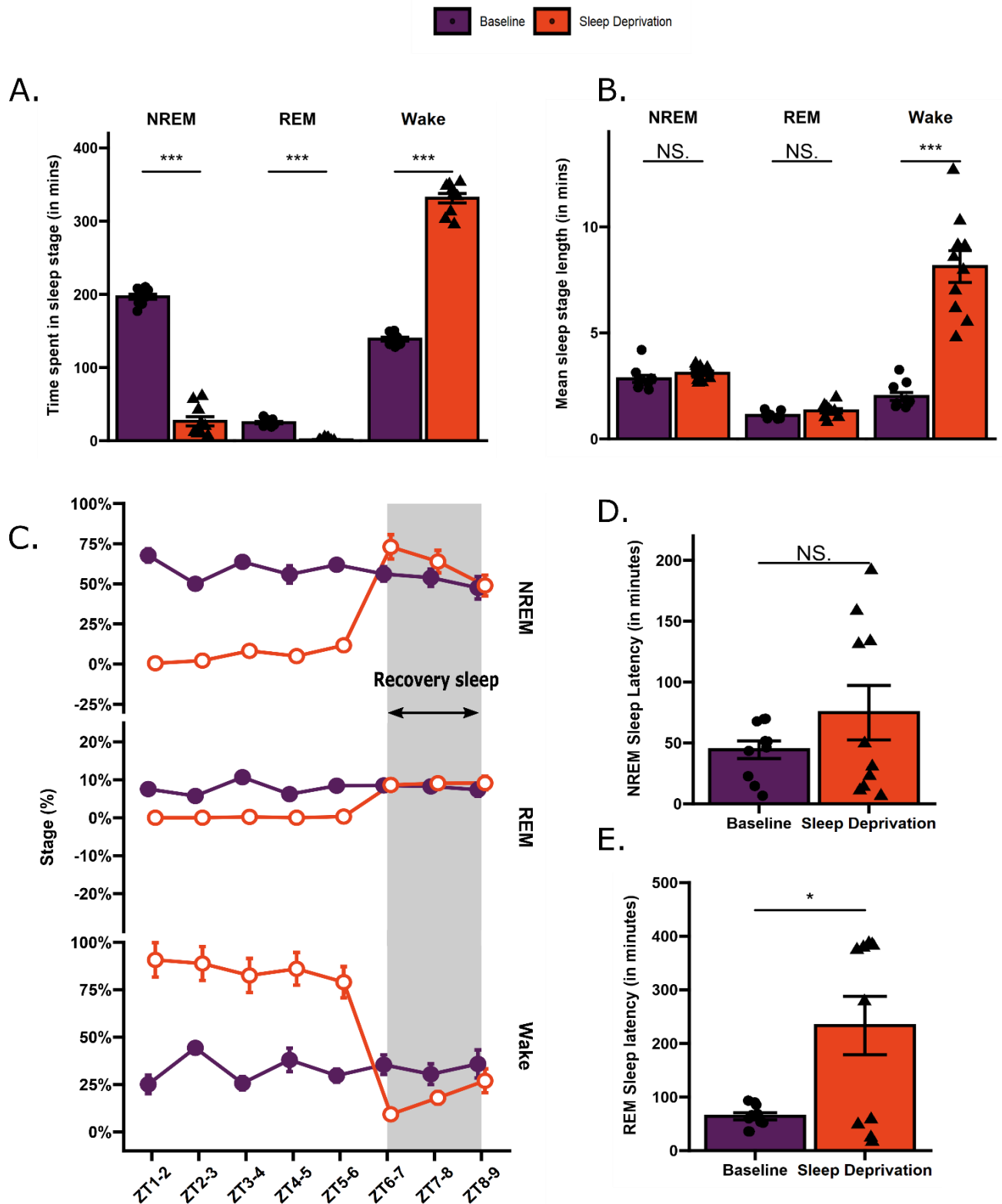
Figure 40: Confirmation of acute sleep deprivation
Sleep deprivation significantly reduced the time spent in each sleep stage (**A**; $n = 10$; all $p < 0.001$) and increased the length of wake bouts (**B;** $t_9 = -7.86$; $p < 0.001$). **C** shows the percentage of time spent in each sleep stage per hour of the recording period. REM (**D**; $t_9 = 4.67$; $p = 0.013$) but not NREM (**E**; $t_9 = 5.82$; $p = 0.190$) sleep latency was significantly increased in sleep deprived mice. Abbreviations: NREM = non-rapid-eye-movement sleep; REM = rapid-eye-movement sleep. Data are represented as mean ± SEM. Shapes overlaid on bars represent data from each individual animal. Comparisons were computed using paired Student's t-tests. * = $p < 0.050$, *** = $p < 0.001$, NS. = not significant at $p < 0.050$.
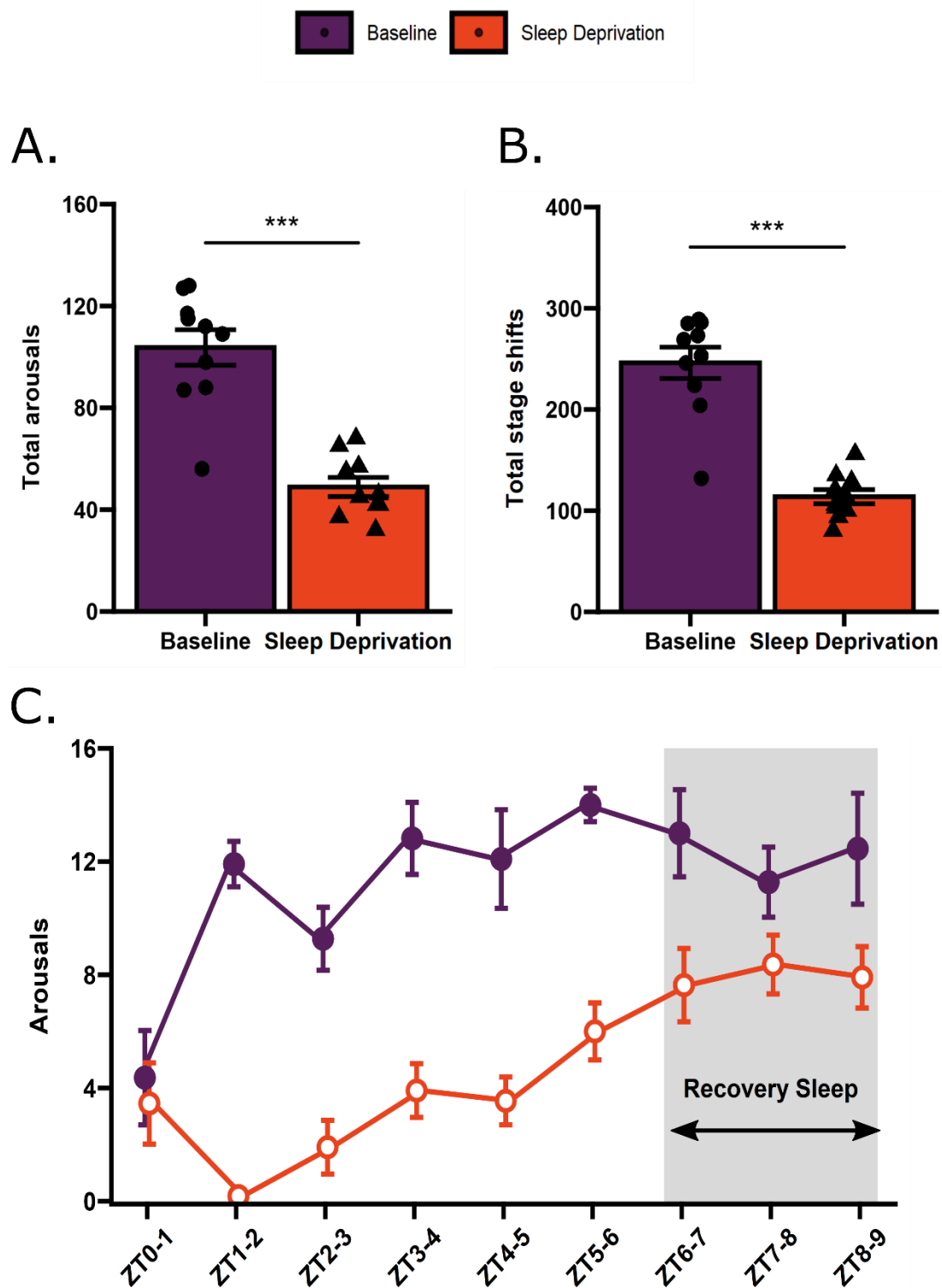
Figure 41: Reduced arousals and stage shifting during sleep deprivation

During sleep deprivation there were significantly less arousals (**A**; t_9 = -1.34; $p < 0.001$; $n = 10$) and stage shifts (**B,** t_9 = 7.13; $p < 0.001$). **C** demonstrates the average number of arousals per hour over the recording period. Abbreviations: NREM = non-rapid-eye-movement sleep; REM = rapid-eye-movement sleep; SD = sleep deprivation. Data are represented as mean ± SEM. Shapes overlaid on bars represent data from each individual animal. For EEG data, comparisons were computed using paired Student's t-tests. For plasma corticosterone, comparisons were computed using mixed ANOVA. * = $p < 0.050$, ** = $p < 0.010$, *** = $p < 0.001$, NS. = not significant at $p < 0.050$.
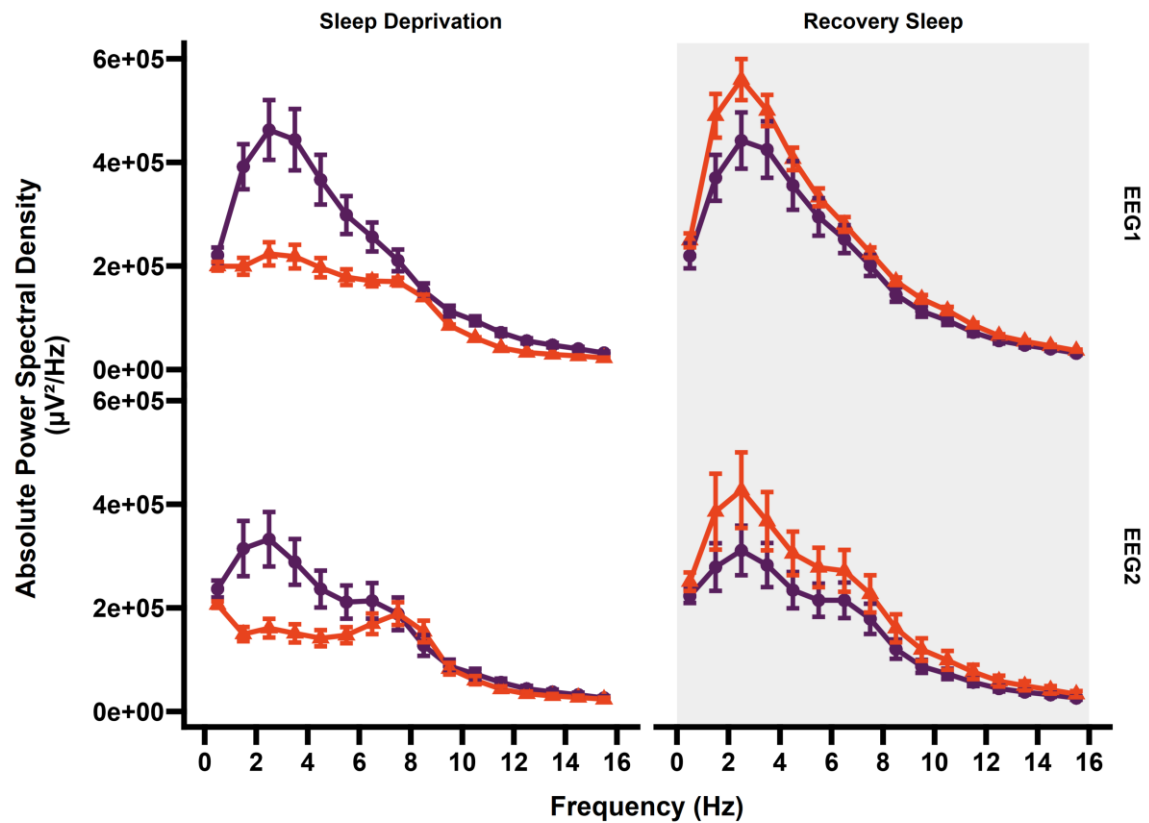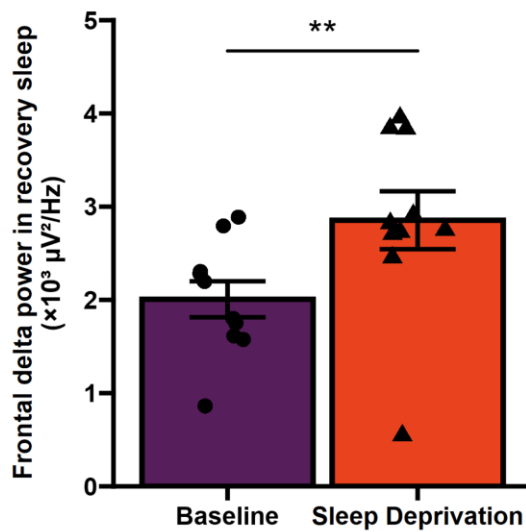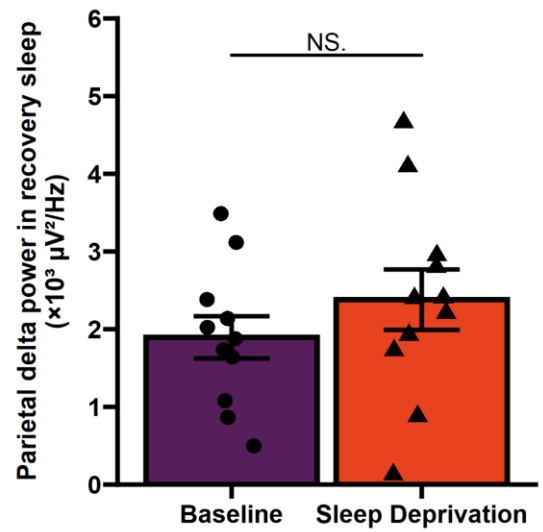
Figure 42: Significant sleep rebound following acute sleep deprivation
Welch's periodograms displaying absolute power spectral density (PSD) during baseline sleep (purple; circles) conditions or during acute sleep deprivation (red; triangles) on two EEG channels: EEG 1 = frontal lobe, EEG 2 = parietal lobe (**A**; n = 10). This is shown both during the sleep deprivation period (ZT0–ZT6) and sleep recovery period (ZT6–ZT9). There is a significant increase in relative PSD in the delta band (1-4 Hz) during the recovery sleep period measured on the frontal EEG channel (**B**; t_9 = -3.32, p = 0.009) but not the parietal EEG channel (**C**; t_9 = -1.86, p = 0.096). Data represent mean ± SEM. Shapes overlaid on bars represent data from individual animals. Comparisons computed using paired Student's t-test. ** = $p < 0.010$, NS. = not significant at $p < 0.050$.
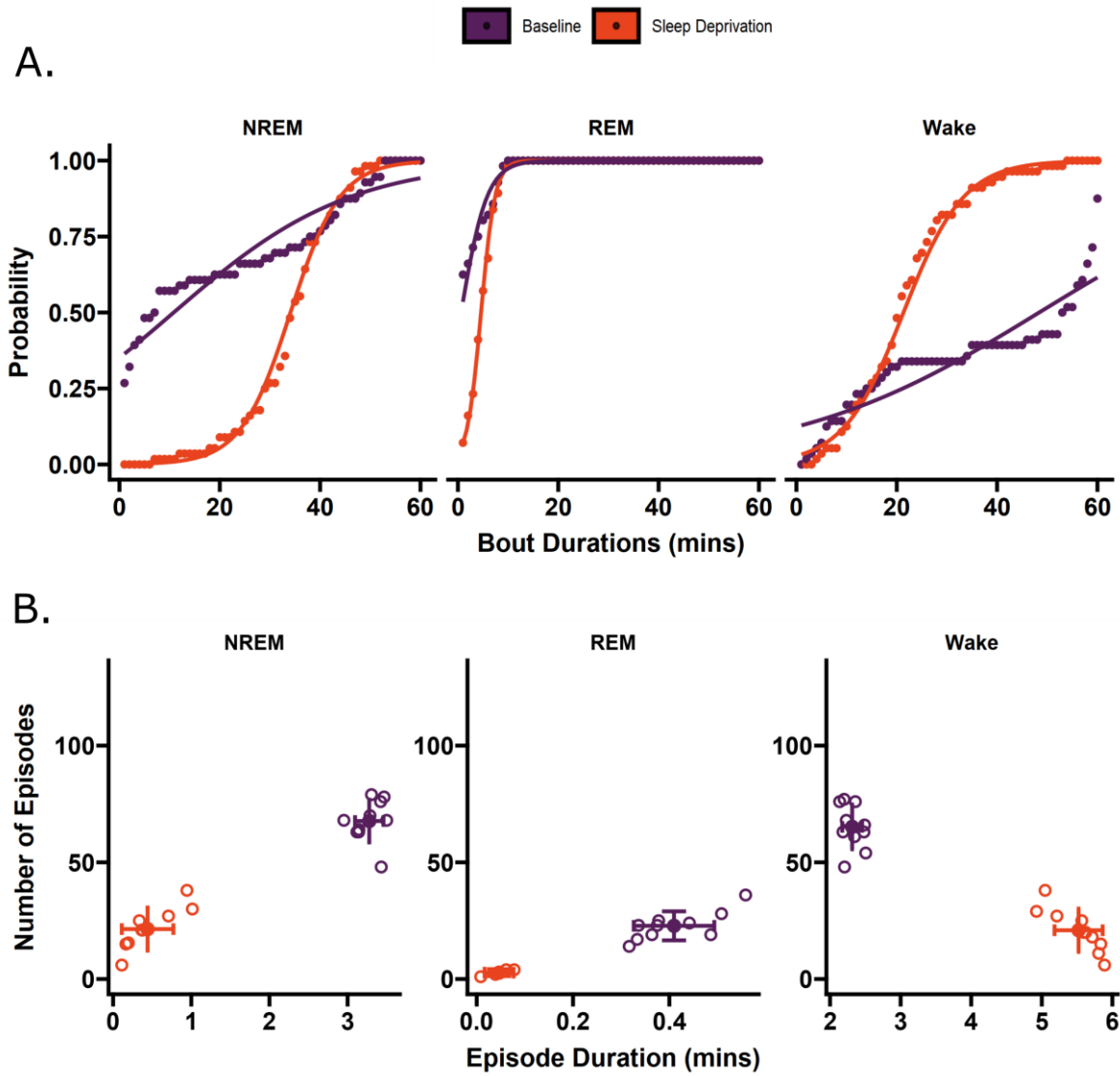
Figure 43: Stage switching and episode duration during sleep deprivation
A) Cumulative distributions for NREM, REM and wake bout durations: the probability of the mouse switching sleep state given a certain bout duration of that sleep stage in minutes for both the baseline (purple) and sleep deprivation (red) sessions (n = 10). **B)** Visualisation of the number of episodes of each sleep stage plotted against the mean duration of episodes in minutes for both the baseline and sleep deprivation sessions. Abbreviations: NREM = non-rapid-eye-movement sleep; REM = rapid-eye-movement sleep. Circles represent data from each individual animal.

5.3.3 Acute sleep deprivation does not affect thermal sensitivity

We explored thermal sensitivity following acute sleep deprivation. There was no significant difference between sleep deprived mice and non-sleep deprived controls at baseline (**Figure 44;** 10.4 ± 1.35 11.2 ± 1.63; $t_{21.2}$ = 1.28; p = 0.213). However, sleep deprived mice had a significantly decreased threshold post sleep deprivation compared to control mice (10.2 ± 2.18 vs 12.7 ± 2.88; $t_{20.5}$ = 2.43; p = 0.024). However, the lack of difference pre to post sleep deprivation suggests that this group difference is not related to the act of sleep deprivation *per se,* as this effect is most likely driven by the increased latency in the baseline group at the second session, suggesting no impact of acute sleep deprivation on thermal hyperalgesia.
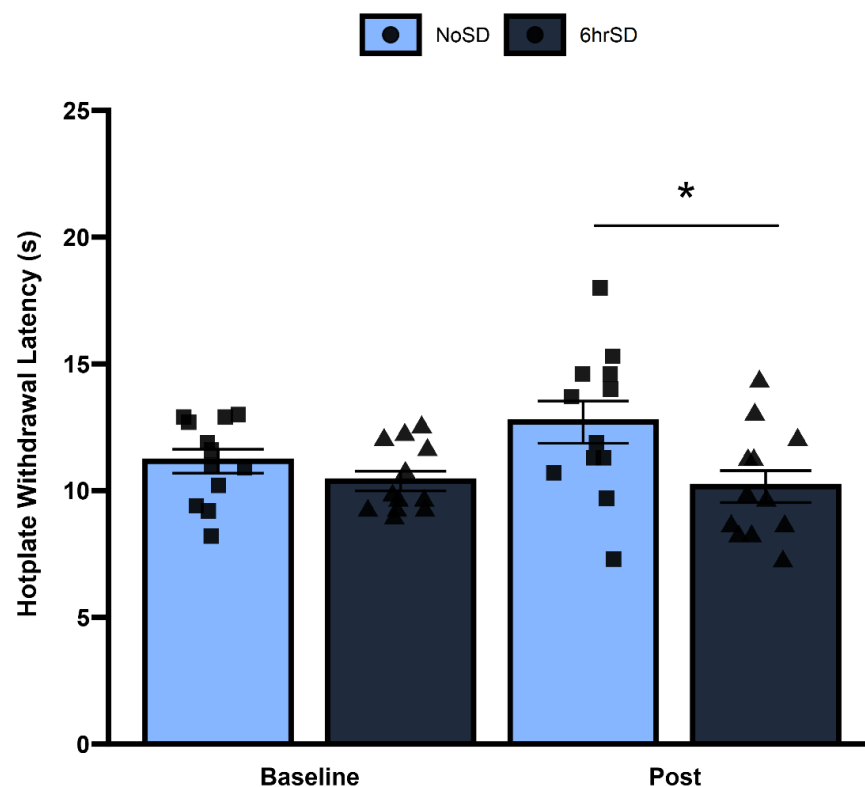
Figure 44: No thermal allodynia following acute sleep deprivation

Thermal sensitivity measured by the hotplate test at baseline and after receiving either six hours of sleep deprivation (6hrSD, n = 12) or no sleep deprivation (NoSD, n = 12). There was no significant difference in latency to evoke a nociceptive response at baseline ($t_{21.2}$ = 1.28; p = 0.213), but there was a significant difference after sleep deprivation ($t_{20.5}$ = 2.43; p = 0.024). Data represent mean ± SEM. Shapes overlaid on bars represent data from individual animals. Comparisons computed using Student's t-tests. * = p < 0.050, NS. = not significant at p < 0.050.

5.3.4 Acute but not chronic intermittent sleep deprivation induces allodynia

To assess if both acute and chronic sleep deprivation affects mechanical hypersensitivity, mechanical thresholds were assessed in the orofacial region. Overall, mechanical thresholds differed between the two sleep deprivation conditions and control ($F(2,21) = 4.10$, $p = 0.032$, $\eta^2_G = 0.16$), and were significantly different across experimental days ($F(3,63) = 3.36$, $p = 0.024$, $\eta^2_G = 0.08$). Thresholds were significantly reduced following six hours of sleep deprivation compared to three hours of sleep deprivation on day one (**Figure 45A**; 0.25 ± 0.20 vs 0.67 ± 0.33; $t_{14} = 4.09$, $p = 0.018$), but not on the other days, or between any of the other conditions (all $p \geq 0.050$).

5.3.5 Sleep deprivation induces c-Fos in the PAG

To explore potential mechanisms, neural activation in key pain structures was investigated following three hours and six hours of chronic intermittent sleep deprivation using c-Fos IHC. Five mice were excluded from this analysis due to inadequate tissue quality (final $n = 19$; 3hrSD = 5; 6hrSD = 7; NoSD = 7). Overall, sleep deprivation affected the number of c-Fos+ cells in the dlPAG (**Figure 46C;** $F(2, 16) = 7.97$, $p = 0.004$, $\eta^2_G = 0.50$), vlPAG ($F(2, 16) = 13.48$, $p = < 0.001$, $\eta^2_G = 0.63$; **Figure 46C**), but not the LH ($F(2,21) = 1.02$, $p = 0.378$, $\eta^2_G = 0.10$; **Figure 46D**).

Six hours of sleep deprivation led to a significant increase in the number of c-Fos+ cells in the dlPAG (13.7 ± 6.85 vs 25.6 ± 11.0; $t_{10} = -2.42$; $p = 0.014$) and vlPAG (9.71 ± 2.93 vs 27.7 ± 13.8; $t_{6.54} = -3.37$; $p < 0.001$) compared to no sleep deprivation. Similarly, compared to three hours, six hours of sleep deprivation increased the number of c-Fos+ cells in the dlPAG (7.6 ± 2.19 vs 25.6 ± 11.0; $t_{6.65} = -4.20$; $p = 0.005$) and vlPAG (3 ± 2 vs 27.7 ± 13.8; $t_{6.35} = -4.66$; $p = < 0.001$). No other comparisons were significant.

5.3.6 Consecutive sleep deprivation induces persistent orofacial allodynia

To assess whether consecutive sleep deprivation without recovery opportunity in-between further exacerbates mechanical hypersensitivity, we assessed thresholds following acute sleep deprivation on three consecutive days (**Figure 45B**). Overall, there was a difference in thresholds across groups ($F(1, 22) = 7.96$, $p = 0.010$, $\eta^2_G = 0.13$), and thresholds were significantly lower across experimental days ($F(4,88) = 2.63$, $p = 0.039$ $\eta^2_G = 0.07$). Post-hoc analysis revealed that as above, mice which were sleep deprived for six hours had significantly lower thresholds than non-sleep-deprived mice on day one (0.25 ± 0.30 vs 0.64 ± 0.49; $t_{18.4} = 2.33$, $p = 0.031$), two (0.25 ± 0.29 vs 0.72 ± 0.56; $t_{16.4} = 2.60$, $p = 0.019$) and three (0.16 ± 0.09 vs 0.62 ± 0.46; $t_{19.2} = 3.43$, $p = 0.005$). However, there was no significant difference in thresholds after recovery sleep (0.58 ± 0.44 vs 0.76 ± 0.44; $t_{22} = 1.03$, $p = 0.315$), suggesting that transient sleep was able to significantly recover this phenotype.
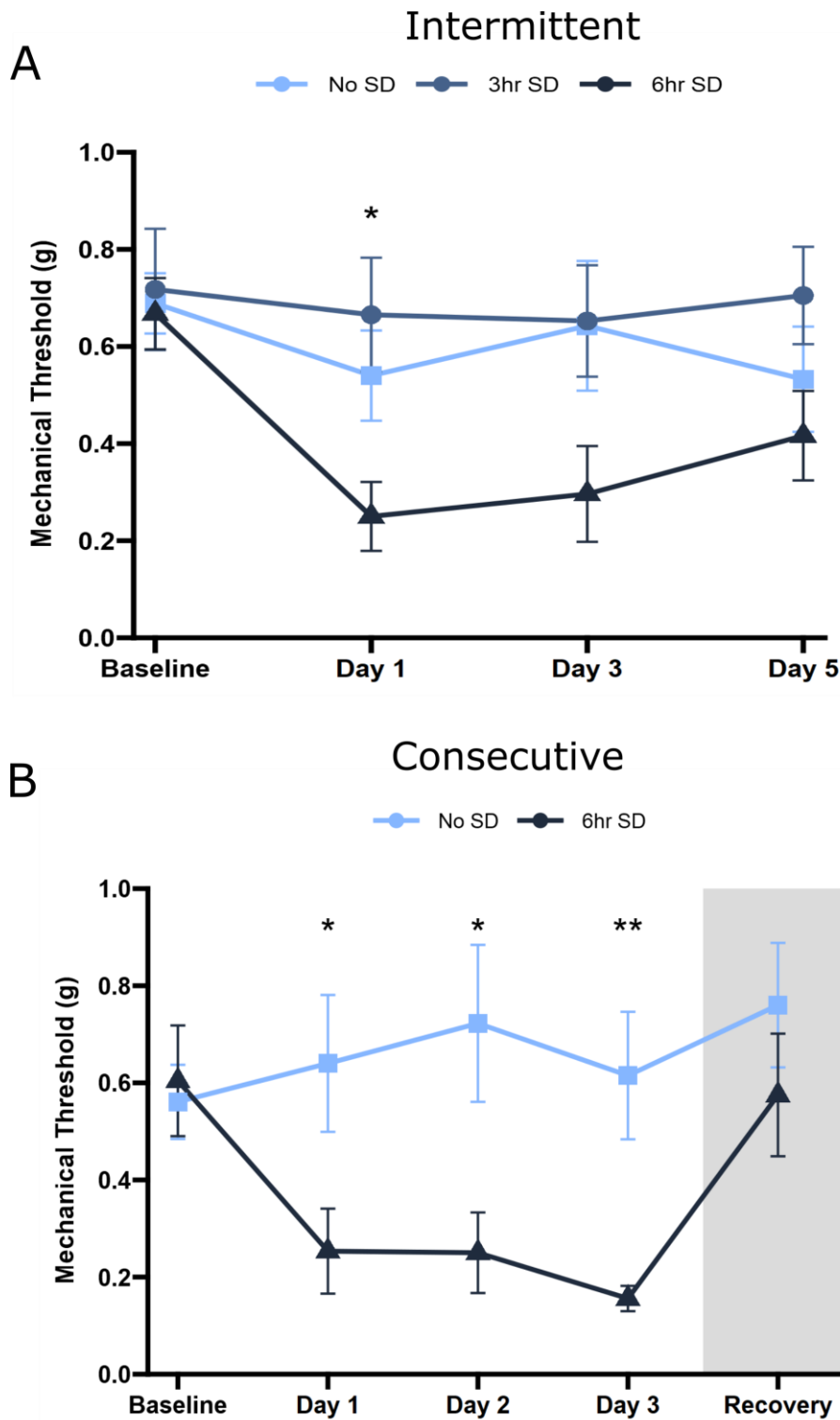
Figure 45: Mechanical hypersensitivity following chronic sleep deprivation
A) Mechanical withdrawal thresholds in grams at baseline, after one, two, and three instances of intermittent sleep deprivation using the gentle handling method, either for three hours ($n = 8$; 3hr SD), six hours ($n = 8$; 6hr SD) or no sleep deprivation ($n = 8$; NoSD). There was a significant difference between mice sleep deprived for three and six hours on day one only ($t_{14} = 4.09$, $p = 0.018$) as assessed by Student's t-test. **B**) There was a significant reduction in mechanical withdrawal thresholds after one ($t_{18.4} = 2.33$, $p = 0.031$), two ($t_{16.4} = 2.60$, $p = 0.019$) and three ($t_{19.2} = 3.43$, $p = 0.005$) instances of consecutive sleep deprivation ($n = 12$ per condition), as assessed by Student's t-test. Thresholds are rescued following three hours of recovery sleep ($t_{22} = 1.03$, $p = 0.315$). Data represent mean ± SEM. Shapes overlaid on bars represent data from individual animals. * = $p < 0.050$, ** = $p < 0.010$.

206
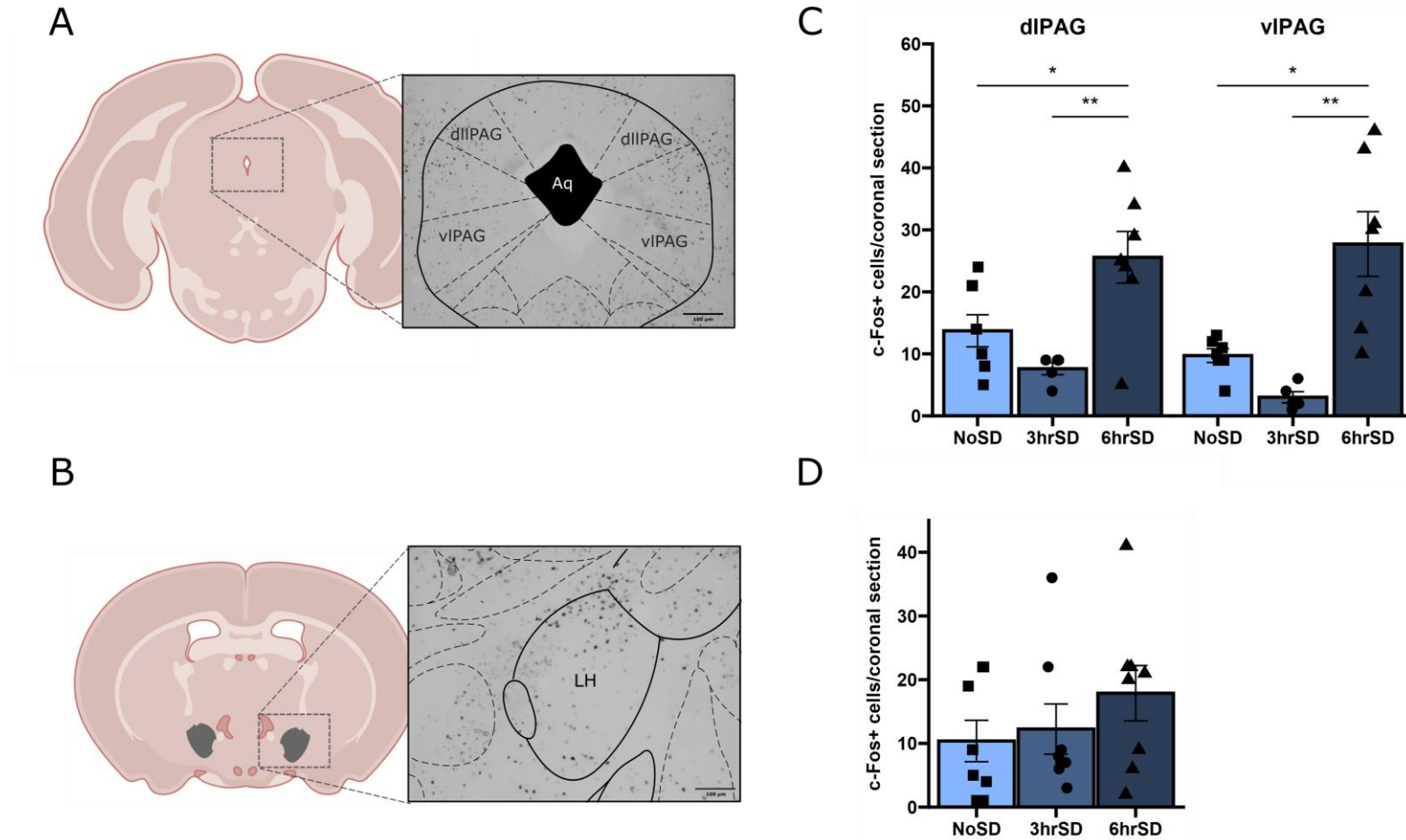
Figure 46: c-Fos+ cells in the PAG and LH following sleep deprivation
A) Indication of the location of coronal PAG slices. **C)** 6hrSD ($n = 7$) significantly increased c-Fos+ cells in the dlPAG ($t_{10} = -2.42$; $p = 0.014$) and vlPAG compared to control ($n = 7$; $t_{6.54} = -3.37$; $p < 0.001$). 6hrSD significantly increased c-Fos+ cells in the dlPAG ($t_{6.65} = -4.20$; $p = 0.005$) and vlPAG ($t_{6.35} = -4.66$; $p < 0.001$) compared to 3hrSD ($n = 5$). **B)** Indication of the location of coronal LH slices, scale bar = 100 μm. **D)** The number of c-Fos+ cells per coronal section in the LH. Sleep deprivation did not affect the number of c-Fos+ cells in the LH ($F(2,21) = 1.02$, $p = 0.378$, $\eta^2 G = 0.10$), as assessed by one-way ANOVA. Abbreviations: vlPAG = ventrolateral periaqueductal grey; dlPAG = dorsolateral periaqueductal grey; LH = lateral hypothalamus. Data represent mean ± SEM. Shapes overlaid on bars represent data from individual animals. * = $p < 0.050$, ** = $p < 0.010$.

5.3.7 Acute sleep deprivation does not affect CGRP expression in the TCC

Lastly, we explored whether acute sleep deprivation led to increased CGRP expression in the TCC. IHC revealed a significant difference in CGRP expression across all groups ($F(2,19) = 5.10$, $p = 0.017$, $\eta^2_G = 0.35$). Post-hoc analysis identified that this was driven by NTG-treated mice, as sleep deprivation did not alter CGRP expression when compared to vehicle control mice (12.5 ± 4.89 vs 11.0 ± 6.15; $t_{12.0} = -0.74$; $p = 0.474$; **Figure 47A-B**). NTG-treated mice had significantly higher CGRP expression than both sleep deprived (16.6 ± 6.29 vs 12.5 ± 4.89; $t_{10.9} = 2.40$; $p = 0.035$) or vehicle control mice (16.6 ± 6.29 vs 11.0 ± 6.15; $t_{12.9} = -2.98$; $p = 0.011$).
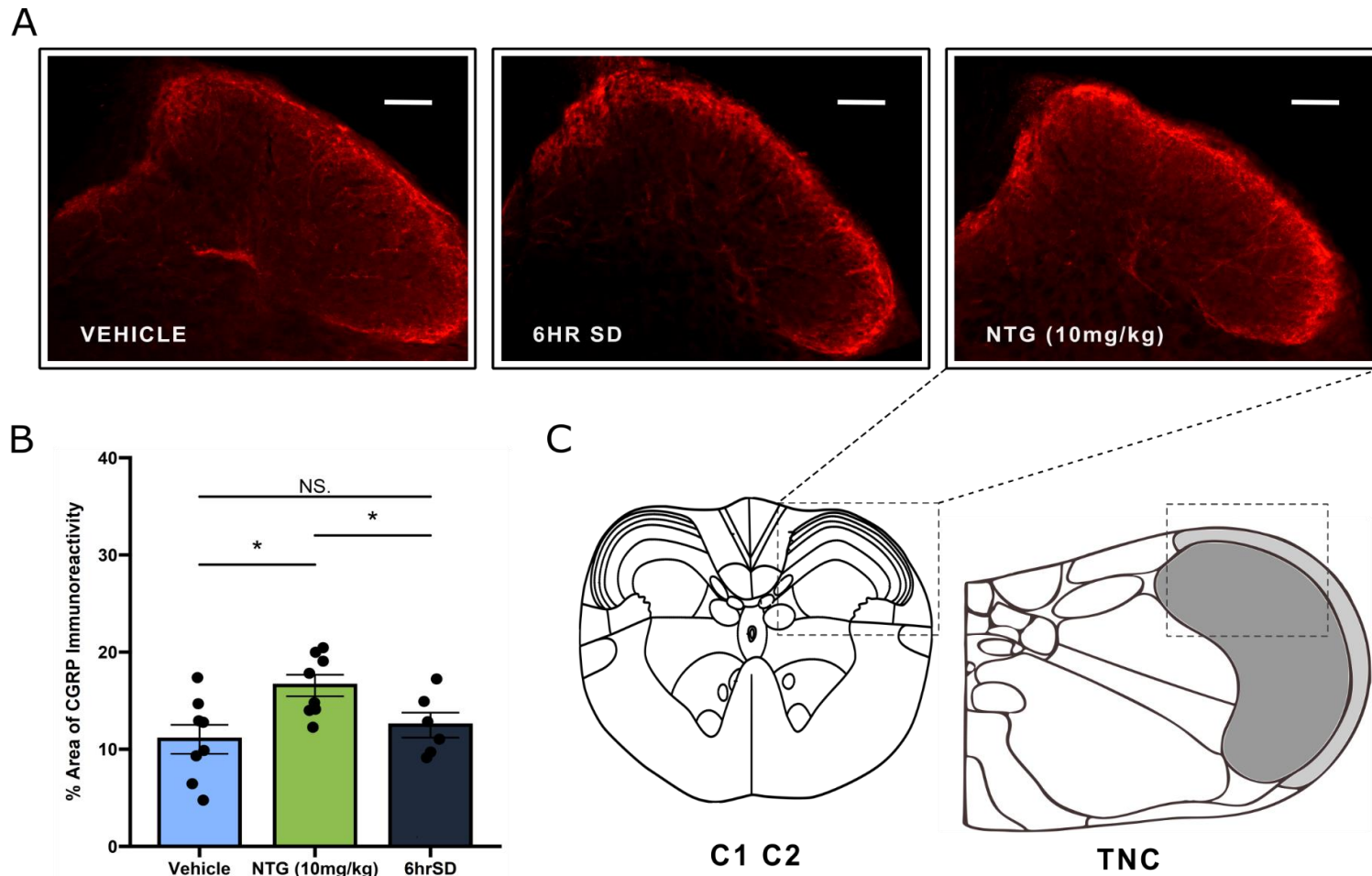
Figure 47: Acute sleep deprivation does not affect CGRP expression in the TCC
A) Representative images taken at 10x of coronal trigeminocervical (TCC) hemi-sections at the trigeminal nucleus caudalis (TNC) level showing CGRP-immunopositive fibres in laminae I and II following i.p. vehicle injection ($n = 8$), 10 mg/kg nitroglycerin (NTG) injection ($n = 8$), or six hours of sleep deprivation (6hrSD) ($n = 6$). **B)** Quantification of the intensity of fluorescent CGRP staining in the TCC. NTG administration resulted in a significantly larger percentage area of CGRP fluorescent staining compared to vehicle ($t_{12.9} = -2.98$; $p = 0.011$), and sleep deprivation ($t_{10.9} = 2.40$; $p = 0.035$) as assessed by Student's t-test. There was no significant difference between sleep deprived mice and those given vehicle injection ($t_{12.0} = -0.74$; $p = 0.474$). **C)** Indication of the location of the images taken from the TNC, C1, and C2 coronal sections which form the TCC. Abbreviations: NTG = nitroglycerin. Data represent mean ± SEM. Circles overlaid on bars represent data points from individual animals. Scale bar = 100 μm; * = $p < 0.050$; NS. = not significant at $p < 0.050$.

5.4 Discussion

Herein, we established that acute and chronic sleep deprivation results in orofacial mechanical allodynia and that this may be mediated by descending pain modulatory structures. More specifically, we found that six, but not three hours of acute sleep deprivation induced orofacial allodynia and elicited neuronal activation in the vlPAG and dlPAG, but not the LH. Brief recovery sleep (three hours) was able to rescue this behavioural phenotype. Only chronic consecutive, rather than intermittent, sleep deprivation was able to induce persistent orofacial allodynia. Lastly, acute sleep deprivation did not elicit thermal extracephalic allodynia, nor increase CGRP expression in areas involved in trigeminal nociception.

Previous studies have largely investigated hind paw hypersensitivity following sleep deprivation, reporting extracephalic allodynia following selective REM sleep deprivation (Kim et al., 2019). Alternatively, studies have demonstrated no extracephalic hypersensitivity following sleep deprivation and fragmentation (Alexandre et al., 2017; Sutton & Opp, 2014). Herein, we reported reduced orofacial thresholds following acute sleep deprivation and chronic consecutive sleep deprivation. Previous evidence for orofacial allodynia following sleep deprivation is limited and conflicting. The only study investigating orofacial thresholds following acute sleep deprivation reported no reduction (Yu et al., 2023).

The discrepancies between previous studies, as well as the current findings, are not surprising. Acute sleep deprivation may have a more pronounced effect on cephalic allodynia than extracephalic. Indeed, previous studies have shown disparities between orofacial and hind paw allodynia in mice after administration of the clinical migraine trigger NTG (Kim et al., 2018). Moreover, previous studies differed in the method they used to measure mechanical allodynia. For example, the outcome can be to measure

the number of withdrawals to von Frey filaments (Alexandre et al., 2017), or the 66% probability threshold (Kim et al., 2019) rather than the 50% threshold used within the current study. Yet, the method of applying the filaments or analysing the data can affect sensitivity (Bonin et al., 2014). Discrepancies could also reflect the different environments and the strain of mice or animal used; von Frey testing has been shown to be sensitive to humidity (Werner et al., 2011), temperature (Andrews, 1993), and angle of impact (Levin et al., 1978).

Intermittent sleep deprivation did not persistently decrease mechanical thresholds, suggesting mice were able to recover from the effects of acute sleep deprivation between sessions. This is surprising as the effects of sleep deprivation on allodynia have been shown to be maintained for up to 96 hours after cessation of sleep deprivation (Hicks et al., 1978, 1979; Kim et al., 2019). Although in the present study we demonstrated that just three hours of brief recovery sleep rescued sleep-deprivation-induced allodynia, corroborating previous reports (Onen et al., 2000; Vanini, 2016). This suggests that consecutive sleep deprivation without recovery opportunity may be required to induce sustained allodynia.

Alternatively, intermittent sleep deprivation when primed with other external trigger factors (e.g. weather, stress) could have more prolonged effects. For example, in the proposed "2-hit priming" model, a triggering stimulus (e.g. stress) induces a state of vulnerability to a later subthreshold stimulus. This is referred to as "latent sensitization" (Kopruszinski et al., 2021). In support of this, one study demonstrated that six hours of acute sleep deprivation did not elicit mechanical allodynia; however, when mice were sensitized with six hours of sleep deprivation prior to NTG administration, this resulted in a prolonged duration of extracephalic allodynia (Yu et al., 2023). This suggests that intermittent sleep deprivation could act as a priming

factor for increased vulnerability to migraine initiation or chronification, which agrees with migraine attacks being influenced by an increasing allosteric load from several factors.

The analgesic effect of recovery sleep aligns with reports of naps relieving attacks in human patients (Blau, 1982), and hypoalgesia observed during and following deep sleep (Callahan et al., 2008). Although, it is unclear whether this is due to sleep *per se* or simply the absence of sensory input which is experienced during sleeping conditions. Furthermore, this could reflect circadian variation in thresholds. Thresholds were measured at ZT10 after recovery sleep, and previous work demonstrated an increase in periorbital mechanical thresholds between ZT6 and ZT12 (Strother et al., 2018). Although, if this were the case, we would have observed a difference in thresholds between day three and recovery sleep in the control group.

In contrast to our hypothesis, we did not observe extracephalic thermal allodynia following acute sleep deprivation. Interestingly, previous research has reported that nine hours of acute sleep deprivation resulted in thermal allodynia in the hind paw (Alexandre et al., 2017), whereas six hours was not sufficient (Yu et al., 2023). Thus, this discrepancy could be related to the duration of sleep deprivation as we conducted six hours, or alternatively could reflect potential separate mechanisms of cephalic and extracephalic allodynia. For example, allodynia is thought to be the result of peripheral sensitisation which first results in cephalic sensitivity before progressing lastly to extracephalic as a result of central sensitisation in 18% of patients (Landy et al., 2004; Mathew et al., 2004). It is possible that although sleep deprivation may evoke extracephalic allodynia with shorter durations of sleep deprivation this may not be apparent.

Despite no reduction in mechanical thresholds on day five of intermittent sleep deprivation, we observed an increase in c-Fos+ cells in the vlPAG and dlPAG. This was partly consistent with our hypothesis and previous literature. For example, previous studies reported an increase in c-Fos expression in the vlPAG, but not in the dlPAG or LH following chronic REM sleep deprivation (Kim et al., 2019). The PAG is involved in modulating nociceptive-related activity in the TCC (Knight & Goadsby, 2001). Thus activation (or inhibition) of the PAG can be pro or anti-nociceptive (Barbaro, 1988; Lee et al., 2000; Lee et al., 2012; Yeung et al., 1978). The current results could reflect a compensatory modulatory mechanism in response to sleep deprivation, but further studies are required to elucidate this.

However, the discrepancy between previous reports of no activation of the dlPAG specifically and the current study could be confounded by stress. For example, the dlPAG can be activated in response to fear and stress (Keay & Bandler, 1993). Although acute sleep deprivation did not increase plasma corticosterone (**Figure 39**), sleep deprivation conducted using the GHM has been shown to induce stress when performed for multiple days suggesting the activation of the dlPAG in our chronic study could be as a result of prolonged sleep deprivation leading to the unmasking of a stress response (Longordo et al., 2011). Conversely, this finding could reflect non-nociceptive activation in the PAG. c-Fos expression is not synonymous with pain or nociceptive processing and neurons can be inhibited after nociceptive stimuli (Harris, 1998). Moreover, the PAG plays an important role in the flip-flop switch between NREM and REM sleep (Sapin et al., 2009). Specifically, inhibition of the PAG reduces REM sleep (Sastre et al., 1996), therefore the activation in the present study may not reflect nociception *per se* but could be an artefact of PAG activation during sleep deprivation (Cirelli et al., 1995).

Surprisingly, and contrary to our hypothesis we observed no increase in c-Fos+ cells in the LH, arguing against a role of the hypothalamus in sleep-deprivation-induced modulation of nociceptive transmission. Previous studies demonstrated an increase in orexin during wake (Kiyashchenko et al., 2002; Taheri et al., 2000) and after sleep deprivation (Estabrooke et al., 2001; Modirrousta et al., 2005), as well as general activation of the hypothalamus in migraine (Denuelle et al., 2007; Maniyar et al., 2014). However, importantly, c-Fos activation does not elucidate which neurons were activated and orexinergic neurons which exclusively reside in the LH express more c-Fos after sleep deprivation, whereas the melanin-concentrating hormone neurons which act to promote sleep in the LH express more c-Fos following sleep recovery (Modirrousta et al., 2005). Moreover, following sleep deprivation NA has been shown to inhibit 90% of orexinergic neurons compared to control conditions (Grivel et al., 2005). Additionally, the hypothalamic peptides OXA and OXB whose cell bodies reside in the LH have been shown to have opposing actions in nociception (Bartsch et al., 2004). Although, the orexin peptides are thought to be expressed and co-released by the same neurons. Thus, specific labelling of orexin receptor expression in sleep deprivation tissue could uncover important differences. However, orexinergic neurons only make up 20% of LH neurons thus other cell types could also be important in modulating allodynia following sleep deprivation.

Lastly, contrary to our hypothesis, we did not observe a significant increase in CGRP in the TCC following acute sleep deprivation. Elevated levels of CGRP are observed in migraine patients (Bellamy et al., 2006; Cernuda-Morollón et al., 2013; Goadsby et al., 1990; Goadsby & Edvinsson, 1993), and administration of CGRP can trigger migraine-like headaches (Hansen et al., 2010). Previous studies have shown an increase in CGRP expression in the cervical spinal cord and TG following NTG

administration (Greco et al., 2018; Wei et al., 1992). We were able to recapitulate the finding of increased CGRP expression in the TCC following NTG administration. This aligns with previous reports of increased CGRP following nitric-oxide donor administration (Wei et al., 1992), but not with other reports of no significant increase in CGRP levels in the TNC following NTG administration (Offenhauser et al., 2005). However, this finding does not rule out increased CGRP signalling following sleep deprivation, but simply demonstrates that there is no significant increase in the TCC. Investigating CGRP levels in the TG and more central regions could provide further information on CGRP signalling and sleep deprivation. The alternative is that sleep-deprivation-induced migraine may not be mediated by CGRP. This is important as this may explain why some patients do not respond to CGRP-based treatments (Ho et al., 2008). Therefore, further investigation of non-CGRP and CGRP-related migraine may be useful for deciding treatment avenues.

The relevance of assessing CGRP expression in the TCC could also be questioned, as CGRP MAbs are thought to act mainly peripherally (Labastida-Ramírez et al., 2023). Future studies could investigate plasma CGRP levels following sleep deprivation to elucidate potential peripheral mechanisms (Ashina, Bendtsen, Jensen, Schifter, & Olesen, 2000). Moreover, using fluorescent intensity to quantify changes in CGRP expression can be affected by methodological factors (Walker, 2006). Whilst measures were taken to mitigate this, such as imaging all sections on the same day, using set exposure parameters, and an automated macro for quantification, future studies could assess neuronal activation via c-Fos expression in the TCC following sleep deprivation as this may be easier to quantify and interpret. We did not undertake this herein due to methodological constraints (e.g. poor quality of spinal cord slices due to ice crystals formed when freezing tissue samples).

5.4.1 Strengths, Limitations & Future Directions:

One strength of this investigation over previous studies is that we were able to confirm in-house the validity of our sleep deprivation method as evidenced by significantly decreased sleep and a sleep rebound during recovery sleep (**Figure 42A**), and no increase in physiological stress (**Figure 39**). Moreover, the application of total sleep deprivation rather than specific deprivation of sleep stages is more translational than previous studies (Hicks et al., 1979; Kim et al., 2019); as humans do not typically experience selective deprivation of REM sleep. Despite these findings furthering our understanding of the link between sleep deprivation and migraine-related phenotypes, there are limitations which should be noted.

One limitation is that we did not explore sex differences due to small sample sizes. Yet, migraine is strikingly more prevalent in females (Victor et al., 2010) and current treatments may have differential efficacy in females versus males (Casteren et al., 2021), and clear sex differences have been observed in response to CGRP in rats (Stucky et al., 2011). Notably, sex differences in response to sleep deprivation have also been observed in mice (Dijk et al., 1989; Koehl et al., 2006), thus future studies should investigate sex differences in allodynia in response to sleep deprivation.

Another limitation is that consideration was not given to the prominent differences between mouse and human sleep. Mice sleep is polyphasic and fragmented, and sleep cycles are much shorter (Chokroverty, 1994; McCarley, 2007). Thus, the impact of total sleep deprivation on naturally fragmented sleep may be different to the consolidated monophasic sleep experienced by humans. Therefore, further investigation into how sleep deprivation in rodents can be translated to humans is warranted (see section 8.4.1 for a detailed commentary on this).

Lastly, whilst we explored mechanical and thermal allodynia in response to sleep deprivation, we did not explore thermal allodynia specific to the orofacial region, nor mechanical allodynia specific to extracephalic regions, therefore future research could compare different modalities of allodynia. Moreover, we focused on allodynia as it is a valid and translational readout in mice (Sureda-Gibert et al., 2022) yet there are other migraine-related symptoms which could be readily explored in mice in response to sleep deprivation including photophobia and CSD (Harriott, Strother, et al., 2019; Harriott, Takizawa, et al., 2019). In fact recent studies reported a reduction in CSD threshold following acute sleep deprivation (Kilic et al., 2018; Negro et al., 2020), highlighting the value of investigating these measures.

5.4.2 Conclusions

In conclusion, we demonstrated that acute sleep deprivation results in mechanical cephalic but not thermal extracephalic allodynia. Furthermore, consecutive, but not intermittent, chronic sleep deprivation results in sustained mechanical allodynia, and brief recovery sleep is able to reverse this phenotype. The mechanisms of this could be mediated by descending pain-modulatory regions.

Chapter 6: Recovery of Migraine-related Phenotypes

6.1 Introduction

In the previous chapter it was established that sleep deprivation induces orofacial allodynia in mice – a commonly reported migraine symptom in patients, and that brief recovery sleep (three hours) can recover this phenotype. In the current chapter, we explored whether this sleep-deprivation-dependent hypersensitivity could be reversed pharmacologically, to identify potential novel therapeutic targets for migraine. This is important as some migraine patients do not respond to treatment (de Boer et al., 2023), indeed the current evidence suggests that 50% of patients respond approximately 50% of the time, and as such there is a need to identify novel pharmacological targets in order to develop more effective and tolerable treatments (Ashina et al., 2021). We also identified that recovery sleep may reverse this phenotype. Whilst improving sleep may be an attractive and simple target for headache, given modern lifestyles including shift work and jet lag, targeting sleep is not always a viable option for patients. Moreover, as sleep disorders and migraine are highly comorbid, insight into the mechanisms of sleep-deprivation-induced increased migraine susceptibility is valuable to further our understanding of migraine pathophysiology.

As detailed in section 1.7.2 the hypothalamus has recently emerged as a key player in migraine attack generation (May & Burstein, 2019; Stankewitz et al., 2021). This is due to the observation of activation during the early premonitory and attack phases (Denuelle et al., 2007; Maniyar et al., 2014; Schulte & May, 2016; **Figure 48**), and perturbed inter-ictal functional connectivity (Coppola et al., 2020; Stankewitz et al., 2011). Interestingly, different hypothalamic nuclei may be involved in episodic versus chronic migraine (Schulte et al., 2017). For example, the posterior hypothalamus is active in response to a noxious stimulus during the acute pain stage,

yet the anterior hypothalamus is more active in chronic migraine patients. The hypothalamus is involved in homeostatic functions such as regulating sleep, blood pressure, heart rate, hormone secretion, and appetite. Alterations in some of these functions are apparent during all phases of migraine (Maniyar et al., 2014; Montagna, 2006; Saper, 2002), including food cravings, thirst, and abnormal tiredness. Deep brain stimulation of the hypothalamus has been shown to be effective in both migraine (Schwedt, 2009) and cluster headache (Láinez & Guillamón, 2017). Moreover, the hypothalamus exhibits diverse projections to major pain-related nuclei such as the LC, PAG, and lamina I of the spinal cord (Bingham et al., 2001; Chiou et al., 2010; Giesler, 1995; Pol, 1999; Robert et al., 2013), in turn, receiving direct nociceptive input through the trigeminohypothalamic tract (Malick et al., 2000). These findings highlight the hypothalamus' potential involvement in migraine pathophysiology.

Figure 48: Hypothalamic activation during spontaneous migraine attacks
Figure taken from Denuelle et al. (2007). This shows the areas of significant regional cerebral blood flow increases (yellow) superimposed on a T1-weighted MRI anatomical reference, highlighting an increase in the hypothalamus.

Within the hypothalamus, the LH is the primary source of the orexin neuropeptides which play a key role in sleep/wake regulation as well as a postulated role in nociception. As discussed in detail in section 1.7.3 there are two orexin peptides: OXA and OXB which are cleaved from the precursor protein prepro-orexin (Sakurai et

al., 1999). Systemic administration of OXA has been shown to have antinociceptive effects in various pain models (Holland et al., 2005; Inutsuka et al., 2016; Razavi & Hosseinzadeh, 2017; Suyama et al., 2004; Yamamoto, Saito, Shono, Aoe, et al., 2003). Conversely, OXB was demonstrated to exhibit pro-nociceptive properties (Bartsch et al., 2004), when administered direct into the posterior hypothalamic area. Therapeutic compounds targeting the orexinergic system have been successful in preclinical migraine models (Hoffmann, Supronsinchai, et al., 2015; L. C. Strother et al., 2018) as well as for insomnia. For example OXA as well as DORAs have been shown to modulate dural-evoked nociceptive responses in the TCC (Bartsch et al., 2004; Hoffmann, Supronsinchai, et al., 2015). DORAs have been shown promise in treating primary insomnia through combating arousal (Xue et al., 2022). Given insomnia and migraine are highly co-morbid (Chu et al., 2021), this suggests a common link between migraine, insomnia, and the orexinergic system. OXA and activation of the OX_1R has also been shown to specifically reverse mechanical allodynia (Cheng et al., 2003; Yamamoto, Saito, Shono, Aoe, et al., 2003; Yamamoto, Saito, Shono, & Hirasawa, 2003).

Given the antinociceptive potential of OXA, we sought to establish whether acute administration of OXA could reverse the orofacial mechanical allodynia previously observed following sleep deprivation in mice (see **Figure 45**). Furthermore, as the analgesic effects of OXA may be dependent on the modality of pain we sought to investigate whether any antinociceptive effects of OXA on mechanical allodynia were specific to sleep-deprivation-induced allodynia. For example, administration of the clinical migraine trigger NTG results in mechanical allodynia (Moye & Pradhan, 2017). Previous studies have shown an analgesic effect of OXA administration or inactivation of the OX1R on NTG-evoked migraine phenotypes including photophobia (Askari-

Zahabi et al., 2022) and thermal allodynia (Kooshki et al., 2020). Therefore, we sought to extend these findings and determine the impact of OXA administration on NTG-evoked mechanical allodynia.

Another important consideration is that the orexins are arousal-promoting neuropeptides involved in wake-stabilisation (Fuller et al., 2006; Piper et al., 2000). Since the von Frey assay used to measure mechanical hypersensitivity relies on subjective observation of mouse behaviour (Callahan et al., 2008), and more active mouse strains are reported to display lower mechanical withdrawal thresholds (Siegfried et al., 1980) we aimed to explore whether any effects of OXA might be explained by heightened arousal levels rather than analgesia. To this end, we employed caffeine – a key arousal-promoting stimulant as a positive control.

Caffeine is an A_1 and A_{2A} receptor antagonist (Fredholm, 1995; Reichert et al., 2022; see section 1.7.3) used to combat subjective sleepiness. Caffeine was chosen rather than other stimulants such as modafinil as it has a complex interaction with headache and is widely used by migraine patients and non-migraine sufferers alike (Nehlig, 1999) and thus including caffeine could also help to further our understanding of its effects on migraine-related phenotypes. Caffeine is often used as an analgesic to treat headache (Lipton et al., 2017), but is also implicated as a precipitating factor (Scher et al., 2004) and may facilitate migraine chronification (Bigal & Lipton, 2006b). However, this is most likely related to withdrawal from caffeine, rather than acute use (Alstadhaug et al., 2020). Moreover, caffeine has well-characterised effects on arousal (Wesensten et al., 2004) highlighting it as a suitable arousal-promoting control. Whereas there is a paucity of evidence linking headache and modafinil (Rains et al., 2008), and modafinil has considerable impact on dopaminergic and serotonergic

pathways (Ferraro et al., 2000; Volkow et al., 2009), which could lead to complex off-target effects.

6.1.1 Aims and Hypotheses

In this chapter we aimed to explore whether OXA could recover sleep-deprivation-induced orofacial allodynia and whether this was specific to allodynia induced by sleep deprivation or could be applied to allodynia evoked by the clinical migraine trigger NTG.

We hypothesised based on the findings in the previous chapter that *i*) orofacial mechanical withdrawal thresholds will be significantly reduced in sleep deprived mice; *ii*) and i.p. administration of OXA will recover these thresholds; *iii*) conversely, we hypothesised that whereas caffeine and OXA would both induce hyperlocomotion, caffeine would not significantly recover thresholds in sleep deprived mice. Lastly, *iv*) we hypothesised that OXA administration would significantly increase mechanical thresholds in mice which were previously sensitised with NTG.

6.2 Methods

6.2.1 OXA Dose Optimisation

OXA has a short half-life and whilst it does cross the BBB via simple diffusion, it has poor affinity and for this reason it is typically delivered intracerebroventricularly (i.c.v.; Kastin & Akerstrom, 1999). Although, recent data from our laboratory has demonstrated success in mouse models of migraine with chronic intranasal delivery of OXA (Sureda-Gibert, 2022). However, in our protocol administration needed to be rapid and repeatable prior to sensory testing, and i.c.v. or intranasal would be methodologically challenging to implement in this design. Therefore, we decided to deliver OXA i.p. and thus the optimum dose of OXA for i.p. delivery needed to be

established. As OXA is arousal-promoting (Hagan et al., 1999; Huang et al., 2001), we used increases in locomotor activity as a surrogate readout of dose efficacy. To establish the dose of OXA required to elicit a measurable impact on sleep-wake cycles we tested two different doses of OXA based on the lower and higher range of doses seen in previous literature (Becquet et al., 2019; Öz et al., 2018; Ran et al., 2015; Tunisi et al., 2019).

Specifically, 12 mice were habituated to circadian cabinets for one week as described in section 2.5 to record locomotor activity as a surrogate marker of sleep/wake cycles. Activity patterns were recorded over a 24-hour period. OXA (40 µg/kg, $n = 6$) or vehicle ($n = 6$) were delivered i.p. at ZT6 to correspond to the same circadian time as the end of acute sleep deprivation. Activity patterns were measured using passive infrared sensors for one week to provide a basal measure and then following each dose accordingly. Following a one-week washout period, the same mice which received OXA now received the 100 µg/kg dose, and the same mice which received vehicle, received this again. Activity patterns were recorded for 24 hours post drug administration. The dose which evoked the greatest activity compared to vehicle and therefore greatest impact on sleep-wake cycles would be utilised in subsequent experiments. A 2-way ANOVA was computed with drug and time relative to injection as within-subjects independent variable and locomotor activity counts as the dependent variable.

The data were not normally distributed for both the 100 µg/kg and 40 µg/kg data (Shapiro-Wilk $p < 0.05$). Therefore, a non-parametric ANOVA-type statistic using the *nparLD* package was computed, which is appropriate for repeated measures over multiple time points between two treatment groups, and more robust with small samples than the Wald-type statistic (Brunner et al., 2002). 40 µg/kg did not

significantly impact locomotor activity counts ($F_{10} = 0.39$, $p = 0.531$), suggesting no impact on arousal. However, with the 100 µg/kg dose, OXA-treated mice were significantly more active than vehicle only at 30 minutes post injection (50.8 ± 9.45 vs 19.7 ± 18.9; $t_{8.85} = 2.68$, $p = 0.025$), suggesting that the 100 µg/kg dose of OXA was sufficient to elicit an acute but significant impact on arousal, seen 30 minutes post injection, therefore this dose and timeframe was used for subsequent experiments.
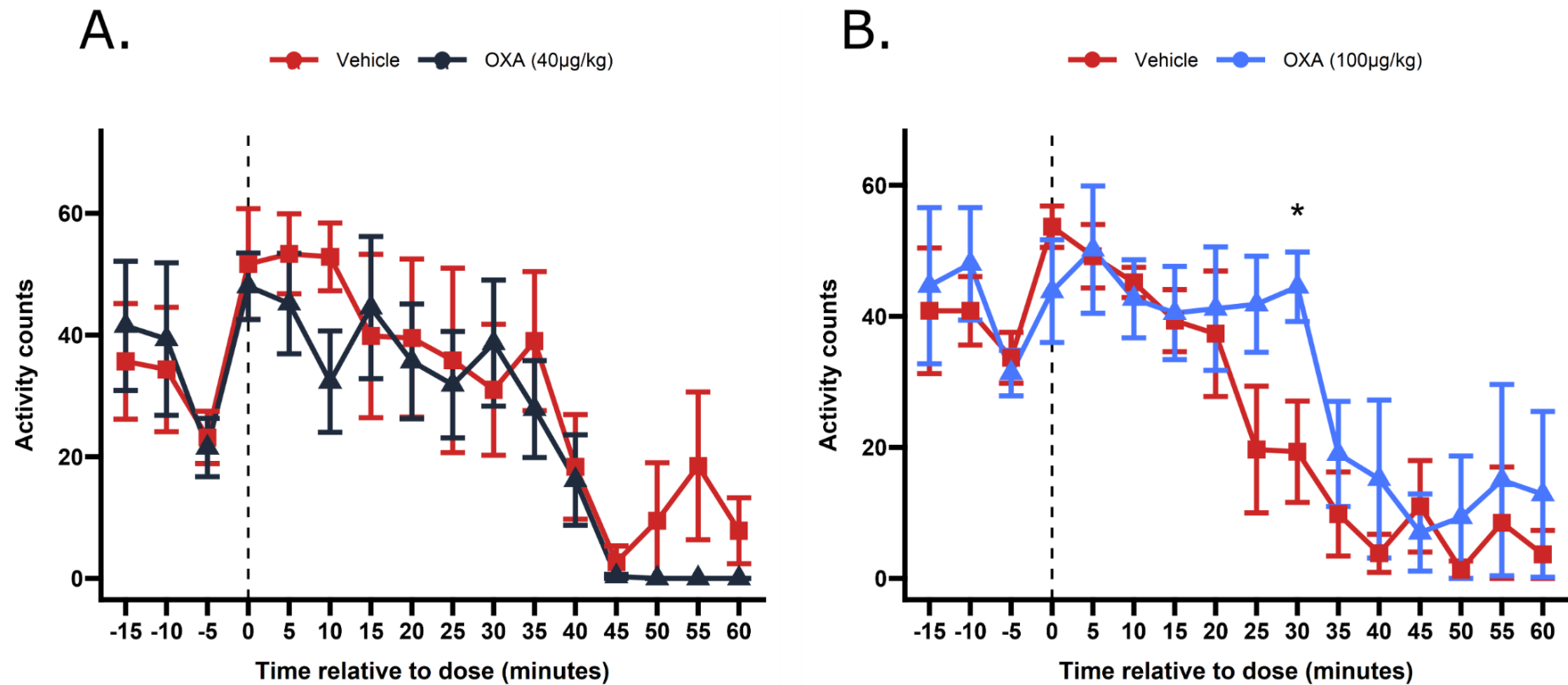
Figure 49: Locomotor activity counts following OXA administration

A) 40 µg/kg of orexin A (OXA; n = 6; triangles) elicited no change in locomotor activity compared to vehicle injection (n = 6; squares). **B)** 100 µg/kg elicited a significant increase in locomotor activity counts compared to vehicle only at the 30 minutes time-point post injection ($t_{8.85}$ = 2.68, p = 0.025) as assessed by Student's t-test. The dashed vertical line represents the time of injection. * = p < 0.050.

6.2.2 Locomotor Activity

As caffeine was chosen as an arousal-promoting control, we needed to first confirm whether our selected doses of caffeine and OXA significantly induced arousal. To do this, the open field arena was used to measure locomotor activity counts. As the open field arena can also be used to test anxiety-like behaviour, mice were habituated twice to the arena as described in section 2.6.3 to reduce any anxiety confounds. 12 mice were counterbalanced to receive either 20 mg/kg of caffeine i.p. (n = 8) based on previous literature (Alexandre et al., 2017), or vehicle (n = 4). Mice were recorded 30 minutes post drug administration based on the pilot study above for eight minutes. Following a five-day washout period, mice which previously received caffeine now received 100 μg/kg OXA (n = 8), and four mice received vehicle. Locomotor activity was recorded at the same time as the sensory testing would be conducted (ZT6), to account for circadian variation in arousal (Silver & LeSauter, 2008). The measures were automatically calculated by the software. As we were interested in analysing locomotion we investigated: the number of gross movements around the arena, and the time spent resting (no movements detected) in seconds in the central (zone 2) or outer zone (zone 1) following administration of each drug. Data were statistically analysed by one-way ANOVA.

6.2.3 Sleep Deprivation

This was a randomised cross-over design. 48 mice (24 male, 24 female) were counterbalanced to receive either six hours of acute sleep deprivation (n = 24), or no sleep deprivation (n = 24) as described in section 2.4. See **Figure 50** for a timeline of the experiment.

6.2.4 Orofacial Mechanical Withdrawal Thresholds

Baseline orofacial mechanical withdrawal thresholds were measured as described in the methods in section 2.6.1. Thresholds were tested immediately after acute sleep deprivation at ZT6 following a 30-minute habituation period to the apparatus. Following this, mice were injected with 100 µg/kg of OXA (n = 12), 20 mg/kg of caffeine (n = 12) or vehicle (n = 24) in two separate cohorts. They were immediately placed back into the apparatus and tested 30 minutes following drug administration as per the pilot study results.

To establish differences between sleep deprived and non-sleep deprived mice at each time point a mixed ANOVA was conducted with mechanical withdrawal threshold in grams as the dependent variable. Where appropriate, Student's t-tests were conducted to interpret main effects using the Bonferroni correction for multiple comparisons. The extreme studentized deviate method (Grubbs' test) was applied to identify significant outliers. Outliers were removed if $p < 0.05$.
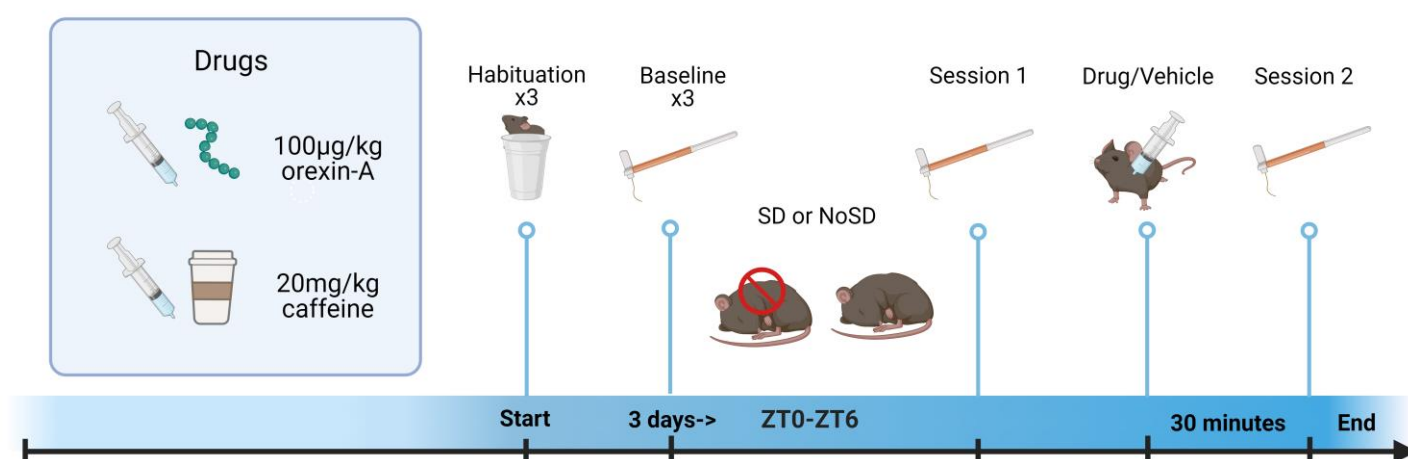
Figure 50: Timeline of the OXA and caffeine experiment
Wild-type mice were habituated three times to the von Frey apparatus and three baseline measurements were taken. Three days later mice were counterbalanced to receive six hours of acute sleep deprivation (SD, n = 12) or left to sleep as usual (NoSD, n = 12). At ZT6 thresholds were tested and immediately afterwards mice were given 100 µg/kg of orexin-A or vehicle intraperitoneal, and in another cohort 20mg/kg caffeine or vehicle. Thresholds were then tested 30 minutes post drug administration. Abbreviations: ZT = Zeitgeber time.

6.2.5 NTG Sensitisation

For this experiment, 24 mice were habituated to the von Frey apparatus and baseline periorbital, and hind-paw mechanical withdrawal thresholds were determined as described previously. Mice were counterbalanced into two groups based on their average baseline thresholds to receive either 10 mg/kg of NTG (n = 12) or vehicle (n = 12) i.p. Two hours post-NTG or vehicle administration, mice were given either 100 µg/kg of OXA (n = 12) or vehicle (n = 12). Thresholds were then tested 30 minutes post drug. Following a one-week washout period, mice were assigned to the same condition as before (NTG/vehicle), however following administration of this drug they were given the opposite drug to the one they received in the previous session (OXA, vehicle). See **Figure 51** for a timeline of the experiment. Thus, each mouse received both treatments (OXA/vehicle).

A mixed ANOVA was conducted with mechanical withdrawal threshold in grams as the dependent variable and where appropriate, Student's t-tests were conducted to interpret this interaction, using the Bonferroni correction for multiple comparisons. The extreme studentized deviate method (Grubbs' test) was applied to identify significant outliers. Outliers were removed if $p < 0.05$.

For all studies, data are reported as mean ± SD, and statistical significance was set at α = 0.05 unless otherwise stated.
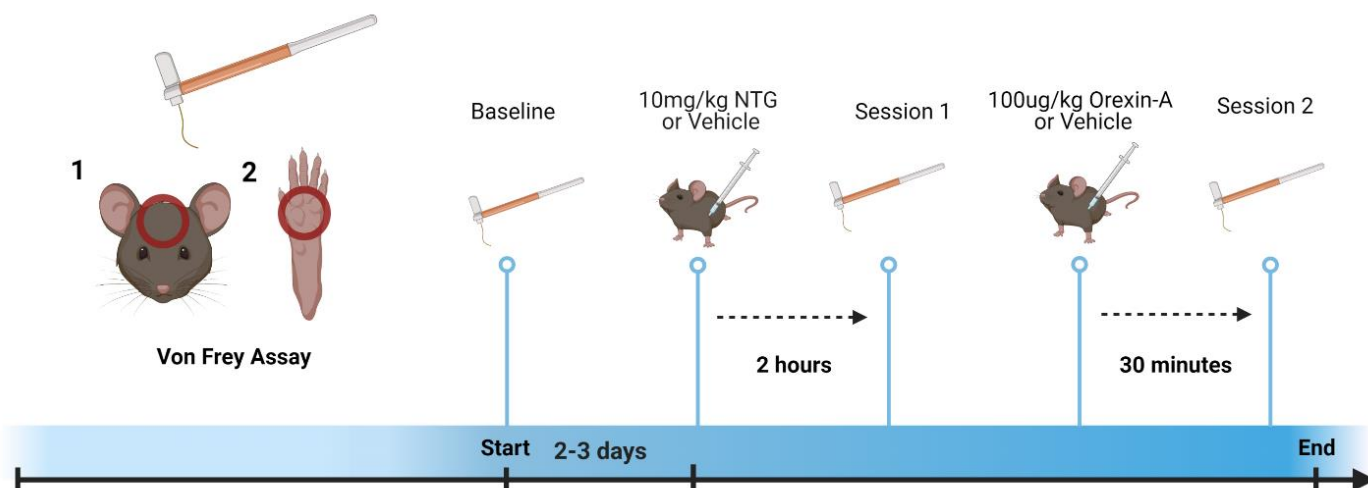
Figure 51: Timeline for the OXA and NTG-evoked allodynia experiment
Mice were tested using the von Frey assay for both the hind-paw and periorbital regions at baseline, two hours after 10 mg/kg Nitroglycerin (NTG) or vehicle i.p. and again 30 minutes following either 100 µg/kg of orexin-A or vehicle injection.

6.3 Results

6.3.1 Caffeine and Orexin-A Increase Locomotor Activity:

To establish whether our doses of caffeine and OXA elicit arousal, we measured locomotor activity counts and time spent in different zones of the open field arena following administration of each drug. Overall, there was a significant main effect of drug on gross movements around the arena ($F(2,14) = 9.51$, $p = 0.002$, $\eta^2{}_G = 0.43$); caffeine and OXA significantly increased movements compared to vehicle injection (1580.50 ± 298.82, $t_{13.9} = 3.10$, $p = 0.023$ vs 1523.25 ± 130.99, $t_{9.92} = 3.59$, $p = 0.015$ respectively), and there was no significant difference between mice which received OXA and caffeine ($t_{9.59} = 0.50$, $p = 0.631$).

Both caffeine and OXA significantly decreased time spent resting in zone 1 compared to vehicle (23.86 ± 17.27, $t_{9.33} = -3.25$, $p = 0.030$ and 29.06 ± 12.40, $t_{8.23} = -3.03$, $p = 0.016$ respectively), with no difference between mice which received OXA and caffeine ($t_{12.7} = -0.69$, $p = 0.501$). No other comparisons were significant.
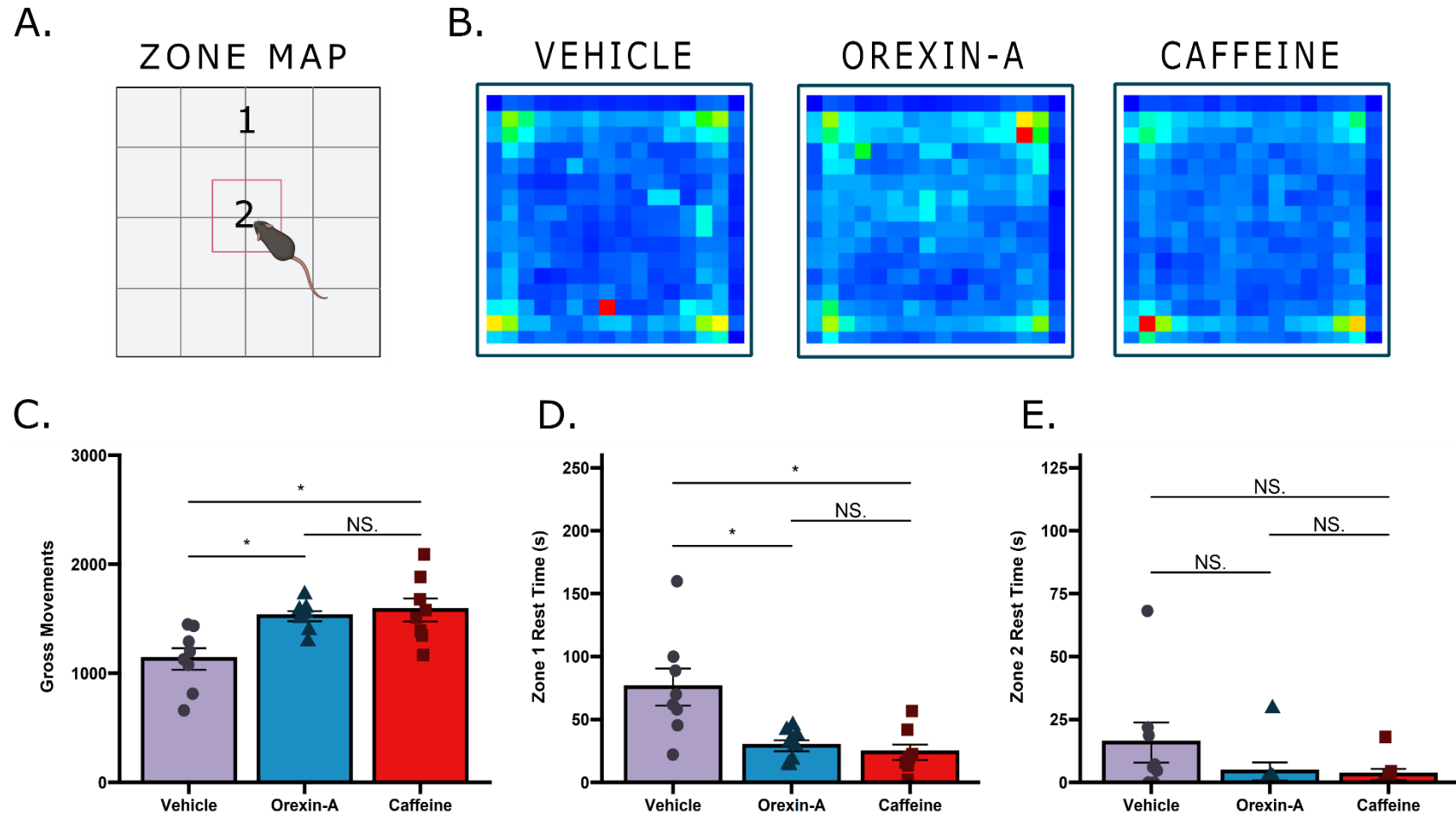
Figure 52: Locomotor activity after administration of caffeine and OXA
A) Schematic of the open field arena. **B)** Heat maps showing the areas of greater activity in the open field arena, red indicates levels of higher activity. **C)** 100 µg/kg of orexin-A ($n = 8$; $t_{9.92} = 3.59$, $p = 0.015$) and 20 mg/kg caffeine ($n = 8$; $t_{13.9} = 3.10$, $p = 0.023$) significantly increased gross movements around the arena compared to vehicle ($n = 8$). **D)** Both caffeine and orexin-A decreased the time spent resting in zone 1 ($t_{9.33} = -3.25$, $p = 0.030$ and $t_{8.23} = -3.03$, $p = 0.016$ respectively), but not in zone 2 (both $p > 0.05$). * = $p < 0.05$, NS. = not significant at $p < 0.05$ as assessed using Student's t-test with the Bonferroni correction for multiple comparisons applied. Data represent mean ± SEM. Shapes represent data points from individual animals.

231

6.3.2 Orexin-A Recovers Sleep-deprivation Induced Orofacial Allodynia:

We sought to explore whether administration of OXA could reverse the mechanical hypersensitivity induced by acute sleep deprivation. Three mice were excluded from this experiment due to failure to habituate to the apparatus and failure to respond to any von Frey filaments (final n = 11 non-sleep-deprived; n = 10 sleep deprived).

As demonstrated previously, sleep deprived mice had significantly lower periorbital mechanical withdrawal thresholds than non-sleep-deprived mice (0.31g ± 0.27 vs 0.55g ± 0.31, $t_{13.6}$ = -2.78, p = 0.045). Administration of OXA significantly increased thresholds in sleep deprived (0.87g ± 0.17, t_{18} = 6.75, p < 0.001; **Figure 53B**) but not non-sleep-deprived mice (0.63g ± 0.35, $t_{19.7}$ = 0.67, p = 0.512; **Figure 53A**), and significantly increased these thresholds above baseline levels (0.87g vs 0.50g, t_{15} = -5.97, p < 0.001). There was no significant difference in thresholds between sleep deprived mice which later received OXA or vehicle (0.50g ± 0.32 vs 0.62g ± 0.11; $t_{17.9}$ = -0.50, p = 0.623), or non-sleep-deprived mice (0.58g ± 0.26 vs 0.45g ± 0.17; $t_{17.6}$ = 0.26, p = 0.800) confirming that the mice which later received OXA/vehicle had comparable thresholds pre-treatment. No other comparisons were significant.
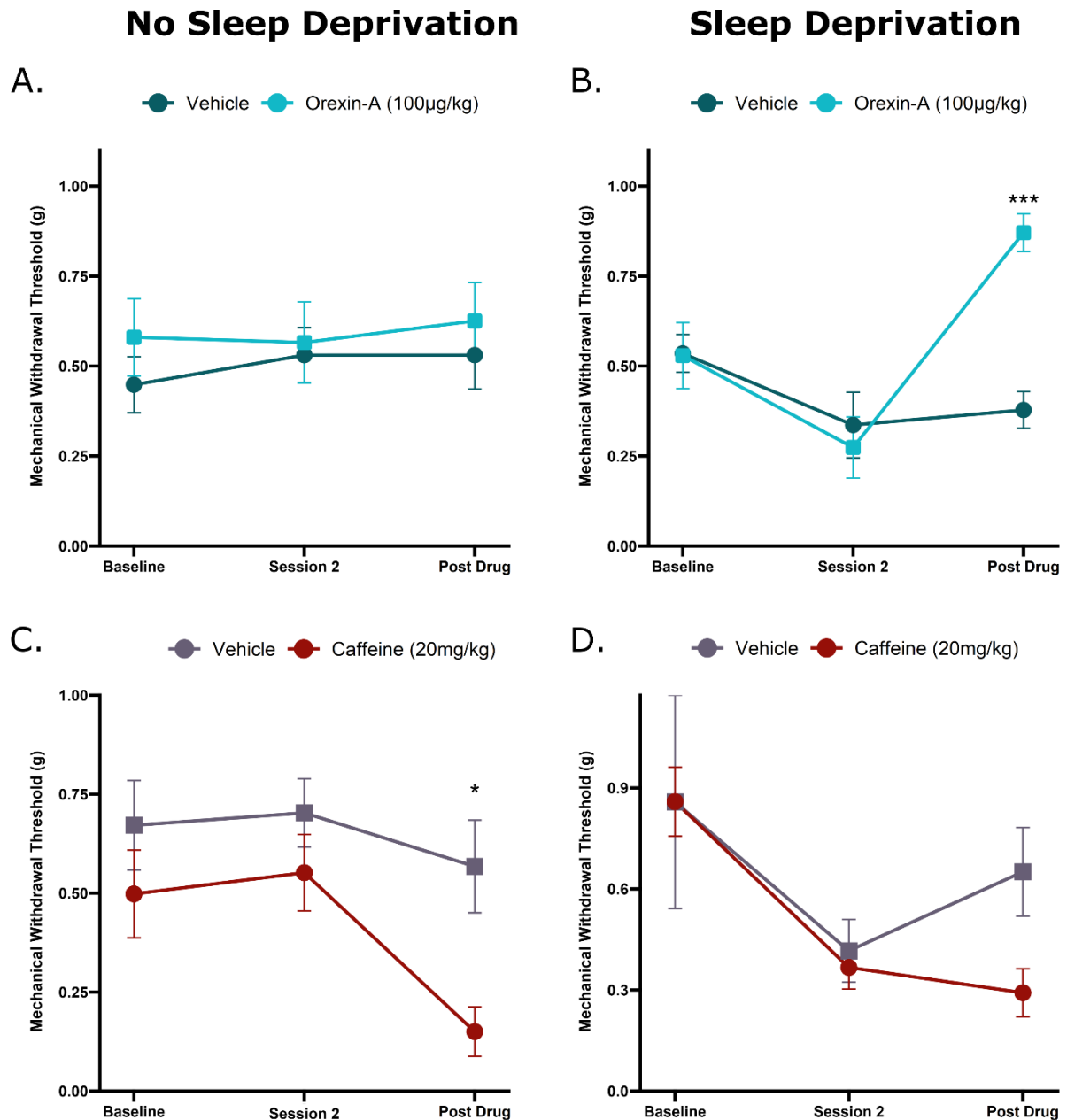
Figure 53: Orexin-A recovers sleep deprivation-induced allodynia
A) 100 μg/kg of orexin-A i.p. (OXA; squares) does not significantly alter mechanical withdrawal thresholds in non-sleep-deprived (non-SD) control mice compared to vehicle (circles; $n = 11/11$; $t_{19.7} = 0.67$; $p = 0.512$). Session 2 refers to the second testing of sensory thresholds following either acute sleep deprivation or being left to sleep as usual. **B)** However, in acutely sleep deprived mice (6hrSD), OXA significantly recovers periorbital mechanical withdrawal thresholds to above baseline levels compared with vehicle ($n = 10/10$; $t_{18} = 6.75$; $p < .001$). **C)** 20 mg/kg of caffeine i.p. (circles) significantly decreases thresholds in control mice compared to vehicle ($n = 12/12$; $t_{16.8} = -3.14$; $p = 0.036$). **D)** Caffeine does not significantly alter thresholds in sleep deprived mice ($n = 12/12$; $t_{17} = -2.40$; $p = 0.168$). Abbreviations: OXA = Orexin-A. Data represent mean ± SEM. *** = $p < 0.001$, * = $p < 0.05$ assessed using Student's t-test with the Bonferroni correction for multiple comparisons applied.

6.3.3 Caffeine Induces Orofacial Allodynia in Non-sleep Deprived Mice:

Secondly, we sought to establish what effect acute caffeine administration would have on mechanical hypersensitivity following acute sleep deprivation. As above, sleep deprived mice had significantly lower periorbital mechanical withdrawal thresholds than non-sleep-deprived mice (0.39g ± 0.27 vs 0.63g ± 0.32, $t_{17.9}$ = -2.75, p = 0.026). Administration of caffeine significantly decreased thresholds in non-sleep-deprived mice (0.15g ± 0.22, $t_{16.8}$ = -3.14, p = 0.036; **Figure 53C**), but not sleep deprived mice (0.29g ± 0.25, t_{17} = -2.40, p = 0.168; **Figure 53D**), thus caffeine did not increase or recover sensory thresholds, and reduced them in non-sleep-deprived animals. There was no significant difference in thresholds between sleep deprived mice that later received caffeine or vehicle (0.37g ± 0.22 vs 0.42g ± 0.32, $t_{19.5}$ = -0.44, p = 0.667), or non-sleep-deprived mice which received caffeine or vehicle (0.55g ± 0.33 vs 0.70g ± 0.30, $t_{21.7}$ = -1.17, p = 0.256), confirming that the mice which later received caffeine/vehicle had comparable thresholds pre-treatment. No other comparisons were significant.

6.3.4 Orexin-A Does Not Rescue Orofacial Allodynia Evoked by Nitroglycerin:

We next aimed to explore whether OXA administration could reverse the periorbital mechanical hypersensitivity evoked by the clinical migraine trigger NTG. Two mice were excluded due to being extreme outliers (Grubbs' test p < 0.05; final n per condition = 11). Mice administered NTG had significantly lower mechanical withdrawal thresholds than vehicle treated mice (0.40g ± 0.23 vs 0.73g ± 0.36; $t_{36.1}$ = -3.52, p = 0.003; **Figure 54A**) as demonstrated in previous studies (Moye & Pradhan, 2017; Pradhan et al., 2014). However, administration of OXA did not affect thresholds in NTG-treated (0.53g ± 0.35; $t_{19.8}$ = 0.17, p = 0.871) or vehicle-treated mice (0.61g ±

0.43; $t_{19.4}$ = 0.21, p = 0.837) compared to vehicle. No other comparisons were significant.

6.3.5 Orexin-A Does Not Rescue Hind paw allodynia Evoked by Nitroglycerin:

Lastly, we observed if the above findings were also observed in the hind paw. Two mice were excluded due to being extreme outliers (Grubbs' test p < 0.05; final n per condition = 11). NTG had a significant impact on hind paw mechanical withdrawal thresholds (F(1,18) = 0.09, p = 0.009, η^2_G = 0.10) as mice administered NTG had significantly lower thresholds than vehicle treated mice (0.40g ± 0.23 vs 0.73g ± 0.36; $t_{35.7}$ = -1.63, p = 0.036; **Figure 54B**). Administration of OXA did not affect thresholds in NTG-treated (0.67g ± 0.39; $t_{19.9}$ = 0.47, p = 0.646) or vehicle-treated mice (0.89g ± 0.37; $t_{18.4}$ = -0.90, p = 0.381) compared to vehicle. No other comparisons were significant.
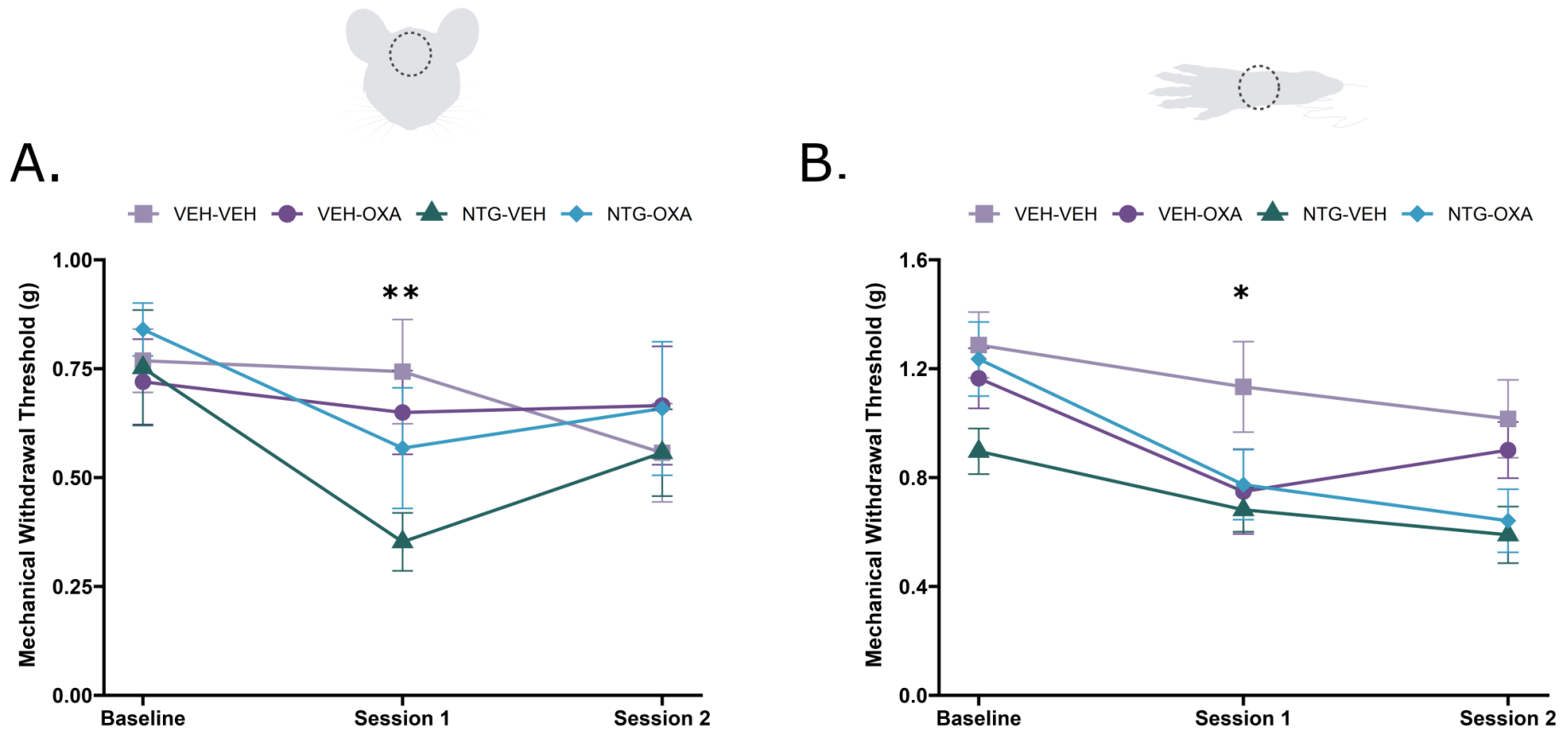
Figure 54: Orexin-A does not rescue allodynia evoked by nitroglycerin
A) Mice administered NTG (n = 11) had lower periorbital mechanical withdrawal thresholds than vehicle treated mice (n = 11; $t_{36.1}$ = -3.52, p = 0.003). However, administration of OXA did not affect thresholds in NTG-treated (diamonds; $t_{19.8}$ = 0.17, p = 0.871) or vehicle-treated mice (triangles; $t_{19.4}$ = 0.21, p = 0.837) compared to vehicle. **B)** Mice administered NTG had lower hind-paw mechanical withdrawal thresholds than vehicle treated mice ($t_{35.7}$ = -1.63, p = 0.036). However, administration of OXA did not affect thresholds in NTG-treated (diamonds; $t_{19.9}$ = 0.47, p = 0.646) or vehicle-treated mice (triangles; 0.89 ± 0.37; $t_{18.4}$ = -0.90, p = 0.381) compared to vehicle. Abbreviations: OXA = orexin-A; VEH = vehicle; NTG = nitroglycerin. Data represent mean ± SEM. * = p < 0.05; ** = p < 0.01 as assessed by Student's t-test with the Bonferroni correction for multiple comparisons applied.

236

6.4 Discussion

The current study aimed to assess whether sleep-deprivation-induced orofacial allodynia could be recovered pharmacologically by the arousal-promoting peptide OXA. The findings indicated that orofacial allodynia induced by acute sleep deprivation can be reversed by administration of OXA. In comparison, caffeine, despite similar arousal promoting effects to OXA, does not recover this allodynia, and induces further sensitisation in non-sleep deprived mice. Therefore, it is possible to speculate that the anti-allodynic effects of OXA are independent of its arousal promoting effects. Surprisingly and contrary to our hypothesis, OXA had no significant effect on cephalic or extracephalic mechanical allodynia evoked by the clinical migraine trigger NTG, in contrast to its previously reported anti-nociceptive effects (Bartsch et al., 2004; Holland et al., 2005) suggesting that the analgesic effects of OXA were constrained to sleep-deprivation-induced allodynia.

In line with our hypothesis and previous reports of antinociceptive effects of OXA (Holland et al., 2005; Razavi & Hosseinzadeh, 2017; Suyama et al., 2004; Yamamoto, Saito, Shono, Aoe, et al., 2003), acute OXA administration recovered mechanical withdrawal thresholds to above baseline levels following sleep deprivation. Interestingly, previous findings have provided conflicting evidence on whether OXA is anti or pro-nociceptive (Razavi & Hosseinzadeh, 2017). The reason for this discrepancy may be that some of the previous studies have injected OXA and OXB at extremely large doses. This means that selectivity for the two orexin receptors (OX_1R, and OX_2R) may be lost, and it may be difficult to disentangle which has pro and which has anti-nociceptive effects (Bigal & Hargreaves, 2013). The method of administration can also impact the results, as OXA has been shown to be antinociceptive when given i.c.v. and intrathecally, but not subcutaneously (Mobarakeh et al., 2005). Efficacy may

also vary based on the interval between administration and testing. OXA only has a short half-life of around 27 minutes when delivered intravenously (Ehrström et al., 2004). Moreover, discrepancies could reflect the time of administration, as endogenous orexin levels exhibit diurnal variation (Grady et al., 2006; Taheri et al., 2000). Interestingly, sleep deprivation elicits increased firing in orexinergic neurons (Verret et al., 2003), as well as changes in the modulation of this firing via noradrenaline, with noradrenaline neurons exerting an inhibiting rather than excitatory effect on the orexin system (Grivel et al., 2005). This could reflect a potential compensatory mechanism of the orexin system in response to sleep deprivation. Further studies are required to elucidate this.

Caffeine was not able to recover thresholds and significantly decreased them in non-sleep deprived mice. As alluded to previously, the effects of caffeine on headache are complex (Espinosa Jovel & Sobrino Mejía, 2017; Nowaczewska et al., 2020). For instance, caffeine was unable to recover thermal hyperalgesia in mice (Ghelardini et al., 2004), and withdrawal from caffeine is associated with improved treatment outcomes in headache patients (Lee et al., 2016). On the contrary, clinical evidence reports an analgesic effect of acute caffeine on headache (Alstadhaug & Andreou, 2019; Migliardi et al., 1994; Ward et al., 1991). Preclinically, Alexandre et al. (2017) found that the same dose and delivery method of caffeine used herein blocked hypersensitivity after chronic sleep deprivation. However, they tested two hours post caffeine administration whereas in the current study thresholds were tested 30 minutes post injection. Yet, mice have been shown to metabolise caffeine at around 45 minutes (Hartmann & Czok, 1980). Moreover, as mentioned previously, cephalic and extracephalic allodynia may require differing durations of sleep deprivation to become apparent – and Alexandre et al. (2017) only investigated hind-paw thresholds.

Another possible explanation of the observed sensitising effect of caffeine is that it is arousal-promoting. If mice are more active, they will likely be more responsive to von Frey filaments (Callahan et al., 2008; Siegfried et al., 1980). Therefore, we could be capturing a sensitising effect of arousal rather than allodynia. An initial stimulating effect of caffeine may occur within the first 45 minutes and an analgesic effect hereafter (Hartmann & Czok, 1980). However, OXA increased locomotor activity to the same extent as caffeine and yet had an opposing analgesic effect, suggesting this is not the case. Moreover, the effects of caffeine on mechanical allodynia are dose dependent, with lower doses (10 mg/kg) increasing allodynia, but higher doses (>50 mg/kg) decreasing allodynia (Tehrani et al., 2020). Therefore, with larger doses, acute caffeine may be analgesic. Moreover, the fact we only observed hypersensitivity in non-sleep-deprived mice could represent a floor effect or limitation of the measurement method, in that mice which are sleep deprived already have low thresholds so lower thresholds were not observable after caffeine administration.

OXA was unable to significantly increase periorbital or hind-paw thresholds in mice sensitised by NTG as opposed to our hypothesis. This was surprising, given its striking effects following sleep deprivation and previous antinociceptive effects of OXA (Holland et al., 2005; Razavi & Hosseinzadeh, 2017; Suyama et al., 2004; Yamamoto, Saito, Shono, Aoe, et al., 2003). For example, research has demonstrated anti-nociceptive effects following intra-vlPAG OXA, and pro-nociceptive effects of OX1R antagonism on NTG-induced photophobia, anxiety, and hyperalgesia (Askari-Zahabi et al., 2022; Kooshki et al., 2020; Pourrahimi et al., 2021). This could also reflect the finding that OXA has been shown to have antinociceptive and pronociceptive effects depending on the method of delivery and experimental procedure (Bartsch et al.,

2004; Holland et al., 2005; Razavi & Hosseinzadeh, 2017; Suyama et al., 2004; Yamamoto, Saito, Shono, Aoe, et al., 2003).

However alternatively, OXA had no significant effect in non-sleep-deprived mice in the first experiment; OXA may only have an effect in sleep deprived mice, suggesting that migraine induced by sleep deprivation may be characteristically different to those induced by other triggers. See below for a more detailed discussion on this.

6.4.1 Potential Mechanisms of Action

In this study, we were able to disentangle the arousal-promoting and nociceptive properties of OXA, as although both caffeine and OXA elicited arousal as measured by an increase in locomotor counts, only OXA had an analgesic action on mechanical allodynia. Regions underlying orexin-mediated analgesia are theorised to also be the site of action for orexin's wake-promoting effects (Ahmadi-Soleimani et al., 2020), suggesting a potential co-regulation of arousal and pain. Other observations support this in that dense orexinergic projections and orexin receptors are widely expressed in both nociception and sleep regulating regions (PAG, LC, DMN, TMN; Peyron et al., 1998). Although it is unlikely that this co-regulation occurs at spinal cord levels.

It is possible that the LH projections to the LC or DMN are involved in the modulation of analgesic effects herein, especially since the LC has highest density of OX1R immunoreactivity (Hervieu et al., 2001), and exhibits both distinct pro and anti-nociceptive subpopulations (Hirschberg et al., 2017). Recent unpublished data from our laboratory demonstrated that injection of OXA directly into the LC was antinociceptive (Sureda-Gibert, 2022). OXA also activates the DMR serotonergic system (Brown et al., 2002; Yamanaka et al., 2003), and effective treatments for migraine work via serotonergic receptors (e.g. triptans), suggesting a possible interplay or orexinergic and serotonergic mechanisms in migraine. Alternatively, OXA

could be exhibiting its analgesic effects via the PAG and endocannabinoid signalling (Lee et al., 2016). For example, the orexins appear to more potent analgesics when delivered i.c.v rather than intrathecally, suggesting a potential supraspinal contribution to anti-nociception, with the PAG being a likely supraspinal target (Chiou et al., 2010). Previous work has shown c-Fos activation in the PAG following orexin administration (Date et al., 1999), and dense orexin receptor expression in the PAG (Marcus et al., 2001). Direct administration of OXA into the vlPAG has been demonstrated to be antinociceptive (Holland et al., 2006; Kooshki et al., 2020; Pourrahimi et al., 2021), and this can be blocked by administration of an OX_1R antagonist into the vlPAG (Askari-Zahabi et al., 2022). However, future tracing studies would be required to elucidate these specific mechanisms and pathways.

An alternative explanation for the dichotomy in arousal and analgesia is that OXA may function in a state-dependent manner depending on the modality of pain. For example, non-sleep-deprived mice treated with OXA, as well as those pre-treated with NTG, did not show a significant increase in mechanical withdrawal thresholds. Previous research suggests that OXA may not be analgesic for acute pain but is analgesic in inflammatory pain models (Bingham et al., 2001), suggesting that the orexinergic system may be involved in pain regulation in a state-dependent manner. Similarly, an OX_1R antagonist (which is selective for OXA; Sakurai & Mieda, 2011) was only pro-nociceptive during inflammatory pain conditions (mouse carrageenan-induced thermal hyperalgesia) but not in acute nociceptive tests (Bingham et al., 2001). Moreover, it is possible that NTG was simply a more noxious stimulus than acute sleep deprivation and therefore required a larger dose of OXA to observe an analgesic effect. This is difficult to tell from the current study as mechanical withdrawal thresholds exhibit a strong floor effect. Alternatively, the intracellular

signalling cascades could simply be different, thus the effects of OXA may be upstream of any effects of NTG.

These results also raise the question of specific orexin receptor involvement. Activation of the OX_2R is postulated to be more arousal promoting than the OX_1R, as dysregulation or knockout results in a striking narcoleptic phenotype (Willie et al., 2003), whereas both receptors are thought to be involved in REM regulation (Mieda & Sakurai, 2009). Whereas the OX_1R is selective for OXA, the OX_2R has equally affinity for both OXA and OXB (Sakurai & Mieda, 2011). Although we did not explore expression of specific receptor sub-types in this study, future studies could investigate this following sleep deprivation and OXA administer thus we cannot be sure via which receptor the analgesic action may be mediated. This would have important considerations for the treatment of migraine by reducing any potential unwanted side effects of orexinergic treatment (e.g. arousal) via activation of the appropriate receptor, and equally, be beneficial for the development of treatments for insomnia disorder, as current treatments target both receptors simultaneously (e.g. DORAs).

6.4.2 Limitations & Future Directions

These findings are not without limitations. Notably, the sleep deprivation method could act as environmental enrichment which has been shown to have analgesic effects in neuropathic pain models (Vachon et al., 2013). However, simple environmental enrichment in mice has been shown to have no effect on mechanical hypersensitivity specifically (Kimura et al., 2019), suggesting that the present antinociceptive effects of OXA are not due to environmental enrichment. Moreover, if this were the case then we would not have expected to see a reduction in thresholds following our sleep deprivation protocol.

Additionally, the effects seen here could be confounded by stress and anxiety. Importantly, anxiety is a key co-morbidity for both migraine and sleep disorders (Leonard, 1994; Peres et al., 2017; Pires et al., 2016). Restraint stress has been shown to induce mechanical allodynia in rats (Bardin et al., 2009), and elicit a pronociceptive state (Robert et al., 2013), and in humans stress can induce or exacerbate allodynia (Crettaz et al., 2013). However, in **Chapter 5** we showed that acute sleep deprivation led to no significant increase in plasma corticosterone levels (**Figure 39**), suggesting that the findings herein are unlikely to be confounded by stress. Similarly, caffeine, especially when taken acutely in non-regular users, can elicit increased cortisol levels (Lovallo et al., 2005). Moreover, OXA administration is linked with increase anxiety (Lungwitz et al., 2012; Suzuki et al., 2005). Future research should investigate measures of anxiety-like behaviours and physiological stress following OXA and caffeine administration.

Another limitation is that we cannot confirm in the current experiments whether the mice were completely sleep deprived as no EEG was conducted simultaneously. However, we have shown previously (section 5.3.2) that sleep deprivation using the gentle handling method significantly reduces both NREM and REM sleep by ~90% of baseline levels. Moreover, we cannot establish exact contributions of specific sleep stages (if any) to orofacial allodynia to further dissect underlying mechanisms. That being said, acute total sleep deprivation is more reflective of sleep deprivation in humans, for example shift work – which is a prominent trigger for migraine (Sandoe et al., 2019) therefore the current results have greater translational value.

6.4.3 Clinical Implications

Whilst these findings demonstrated that OXA can recover orofacial allodynia, there are key considerations when translating these findings to the clinic. A recent trial

employing DORAs (antagonization of both orexin receptors) in migraine patients was negative (Chabi et al., 2015). However, the current findings suggest that increasing OXA rather than dampening orexin may have therapeutic benefits. Indeed, since the work in this thesis was conducted a selective OX_1R agonist has been developed and has been shown to be antinociceptive in mouse models (Iio et al., 2023) further supporting our findings. However, as shown here, OXA has a clear impact on arousal, and chronic delivery could lead to side effects including insomnia, thus further chronotherapy studies should be conducted to ascertain the optimum timepoint of treatment delivery in migraine patients. For example, in this study OXA was delivered during the light phase when mice are typically asleep, yet this could potentially lead to sleep disruption and insomnia thus OXA might be best delivered during wake (Willie et al., 2011). This may be achieved using oral extended-release drug delivery systems. For example, controlled-onset extended-release (COER) verapamil – a calcium channel blocker used in the treatment of cluster headache is specifically designed for bedtime dosing and morning release (Ranganathan et al., 2021). Given that most migraine attacks occur in the early hours of the morning upon awakening (see **Figure 31**), COER systems could be used to deliver OXA-related therapies at night, ensuring less impact of OXA on sleep. COER drugs are already widely used to treat insomnia (e.g. zolpidem; Moen & Plosker, 2006; Moline et al., 2021).

Alternatively, acute OXA could be delivered in the morning. This may have double benefit in that it is arousal promoting, fighting fatigue in the early hours of the morning when most migraine attacks occur. Future studies could also explore the impact of intranasal OXA. Intranasal is a widely used therapy and intranasal CGRP antagonists (von Mentzer et al., 2020) have recently been FDA approved. Intranasal

is a preferable route as it by-passes the BBB (Dhuria et al., 2010) and therefore may more directly target the TG and hypothalamus.

6.4.4 Conclusions

Herein, we determined that acute sleep deprivation elicits orofacial allodynia thus directly highlighting a causal link between sleep and migraine; in that poor sleep triggers migraine-related symptoms. Moreover, we demonstrated that administration of OXA recovers this phenotype, which is likely to be independent of its arousal-promoting properties. OXA should be explored further as a potential underlying treatment for attack resolution with chronotherapy studies to determine the optimum timepoint of delivery.

Chapter 7: Migraine Susceptibility in Short Sleepers

7.1 Introduction

In previous chapters (**Chapters 4-6**) we reported that sleep disruption/deprivation may be a risk factor for an increased susceptibility to migraine attacks. However, in human migraine patients, sleep disturbance is only a reported trigger for around 50% of individuals (Kelman, 2007) and it is unknown why some patients may be less susceptible to sleep-deprivation-induced increased migraine susceptibility. Therefore, the present chapter aimed to explore potential mechanisms underlying susceptibility to migraine.

Whilst short sleep duration is typically a risk factor for disease and increases the likelihood of mortality (Gallicchio & Kalesan, 2009; Itani et al., 2017), research has identified specific individuals as familial natural short sleepers (FNSS) who are less susceptible to the negative consequences of sleep deprivation (see section 1.5.6) . For example, anecdotal reports suggest reduced cognitive difficulties following sleep deprivation, reduced susceptibility to life stressors, and increased pain tolerance in FNSS. Empirically, human FNSS exhibit greater immune function (Fondell et al., 2011) and mice carrying mutations for FNSS show reduced Alzheimer's-related pathology (Dong et al., 2022). Importantly, FNSS are set apart from facultative short sleepers who require more sleep to functional optimally but choose shorter sleep duration due to work or social constraints, and experience sleep-deprivation-induced cognitive difficulties (Yook et al., 2021). The empirical evidence on objective sleep measures in FNSS is lacking, however they have been reported to operate and cope with higher homeostatic sleep pressure than those with conventional sleep length (Aeschbach et al., 1996).

Transgenic FNSS mice have been generated after identification of specific mutations in human NSS families (Zheng & Zhang, 2022). One such mutation is the

P384R point mutation in DEC2 identified in a human family by He et al. (2009). Carriers exhibit reduced sleep duration (4-6 hours) compared with non-carriers (6-8 hours; see **Figure 55**). DEC2 is a transcriptional repressor which belongs to the basic helix-loop-helix family. It is a negative component of the circadian clock and represses CLOCK/BMAL1-induced transactivation through E-box elements (He et al., 2009; **Figure 6**). The P384R mutation changes a C to a G in the DEC2 DNA sequence which is proposed to cause a proline-to-arginine alteration at amino acid position 384. Other mutations have been reported which are linked with short sleep duration and transgenic mouse lines harbouring these mutations have been generated (see **Table 22** for an overview).

The precise mechanisms behind the resulting short sleep phenotype in *h*DEC2-P384R mice are unknown but may be related to greater sleep efficiency and increased SWS depth, as the DEC2 gene is thought to regulate homeostatic sleep drive (Hirano et al., 2018). This notion is supported by an increase in delta power during NREM sleep in *h*DEC2-P384R mice (He et al., 2009). Mice harbouring the mutation were also shown to have increased orexin levels (both peptide and OX_2 receptor mRNA; Hirano et al., 2018) measured in the hypothalamus. Mutant *h*DEC2 is thought to be a weaker transcriptional repressor of transcription factors: MyoD1, E12, and E47 than WT, providing a mechanistic explanation for short sleep given the orexinergic system's role in arousal (see **Figure 56**). Indeed, when mice were treated with an orexin-receptor antagonist (MK-6096; Hirano et al., 2018), this attenuated the decreased immobility time (e.g. increased sleep time) in these mice, thus further confirming the connection between *h*DEC2 and the orexin pathway.
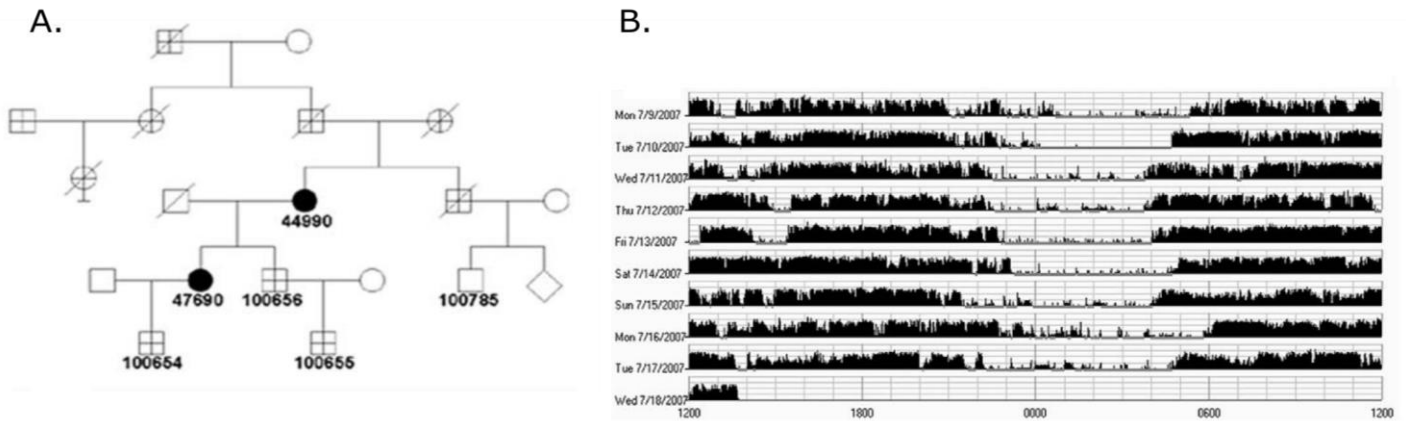
Figure 55: DEC2-P384R mutation in human carriers
A) Pedigree of family containing the hDEC2-P384R mutation. B) Activity recording using actigraphy for one human carrier indicating an extended activity period each day. The x axis represents clock time. From He, Y., Jones, C. R., Fujiki, N., Xu, Y., Guo, B., Holder Jr, J. L., ... & Fu, Y. H. (2009). The transcriptional repressor DEC2 regulates sleep length in mammals. *Science,* 325(5942), 866-870. Reprinted with permission from AAAS and adapted with permission from authors.
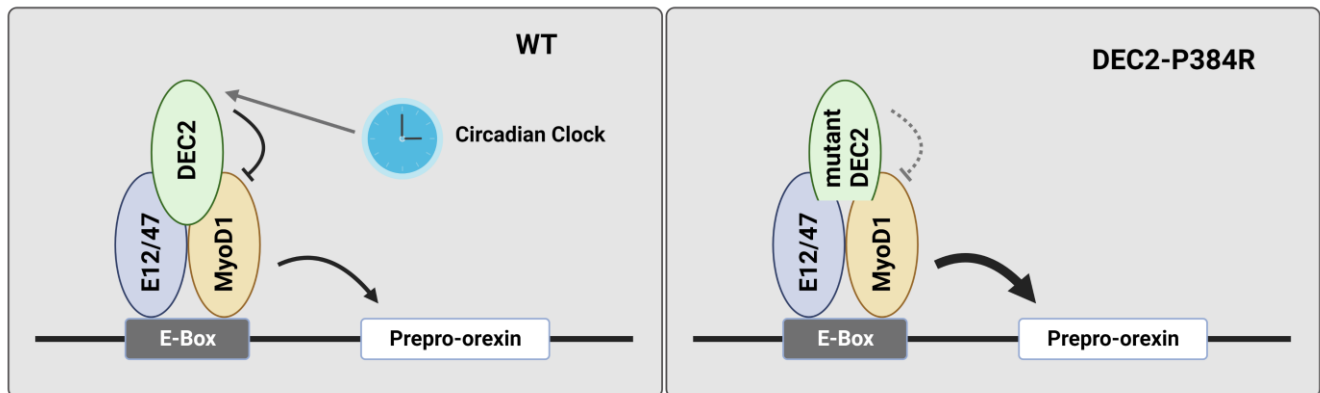
Figure 56: Model for regulation of prepro-orexin expression by DEC2
DEC2 binds and competes with transcription factors (MyoD1, E12, and E47) and suppresses the transcriptional activity of orexin (Left). The P384R mutation in DEC2 decreases the interaction with E12 and E47 leading to up-regulation of prepro-orexin (Right). Adapted from Hirano, A., Hsu, P. K., Zhang, L., Xing, L., McMahon, T., Yamazaki, M., ... & Fu, Y. H. (2018). DEC2 modulates orexin expression and regulates sleep. Proceedings of the National Academy of Sciences, 115(13), 3434-3439. Adapted with permission from the corresponding author.

Of relevance to migraine, previous work from our laboratory and others demonstrated that mutations in circadian clock genes results in heightened sensitivity to migraine-related phenotypes. For example, mice harbouring the mutations hCK1δ-T44A and hPER2-S662G as models of familial advanced sleep phase (FASP) exhibited increased susceptibility to low doses of the migraine trigger NTG, which evokes periorbital hypersensitivity and CSD events under baseline conditions (Brennan et al., 2013; Strother, 2020).

Whilst sleep deprivation in conventional sleepers and alterations in circadian timing may be pronociceptive (Wei et al., 2022), it is possible given anecdotal reports of resistance to sleep deprivation and pain in FNSS, that they may exhibit reduced migraine susceptibility. This could provide important insight into migraine pathophysiology and identification of novel treatments. Indeed, one FNSS mutation: NPSR1-Y206H resulted in potential higher pain thresholds in human carriers (see **Figure 57**). However, investigations with transgenic mice carrying this mutation were negative – there were no significant alterations in thermal or mechanical sensitivity. Given that these mutations result in different sleep phenotypes (**Table 22**) it is possible that other FNSS mutations result in reduced pain susceptibility. Additionally, no migraine-specific readouts (e.g., photophobia, CSD) have been investigated in FNSS.

Therefore, we investigated susceptibility to pain and migraine-specific readouts in mice harbouring a mutation for FNSS. Given that hDEC2-P384R transgenic mice display a smaller sleep rebound in response to acute sleep deprivation and exhibit increased orexin expression (Hirano et al., 2018), and in the previous chapter we reported an analgesic effect of OXA, this transgenic line was the focus of the experiments herein.

Table 22: Comparison of transgenic FNSS mouse lines

FNSS line	TST	NREM	REM	NREM delta power	Sleep rebound	Sleep rebound delta power	Preclinical pain data	Clinical pain data	Reference
Grm1b-R889W	Shorter (light & dark)	Less (light & dark)	No difference	No difference	No difference	No difference	-	Possible increased pain thresholds	Shi et al. (2021)
Grm1-S458A	Shorter (light & dark)	Less (light & dark)	No difference	No difference	No difference	No difference	-	-	Shi et al. (2021)
ADRB1- A187V	Shorter (dark)	Less (dark)	Less (dark)	-	-	-	No difference	-	Shi et al. (2019)
NPSR1- Y206H	Shorter (light & dark)	Less (light & dark)	Less (dark)	Increased compared to WT	No difference	Increase in the 1st hour	No difference	Possible increased pain thresholds	Xing et al. (2019)
DEC2-P384R	Shorter (light)	Less (light)	Less (light)	Increased compared to WT	Smaller sleep rebound	No increase	-	Possible increased pain thresholds	He et al. (2009a)

Abbreviations: FNSS = familial natural short sleeper; TST = total sleep time; NREM = non-rapid-eye-movement sleep; REM = rapid-eye-movement sleep; WT = wild-type.
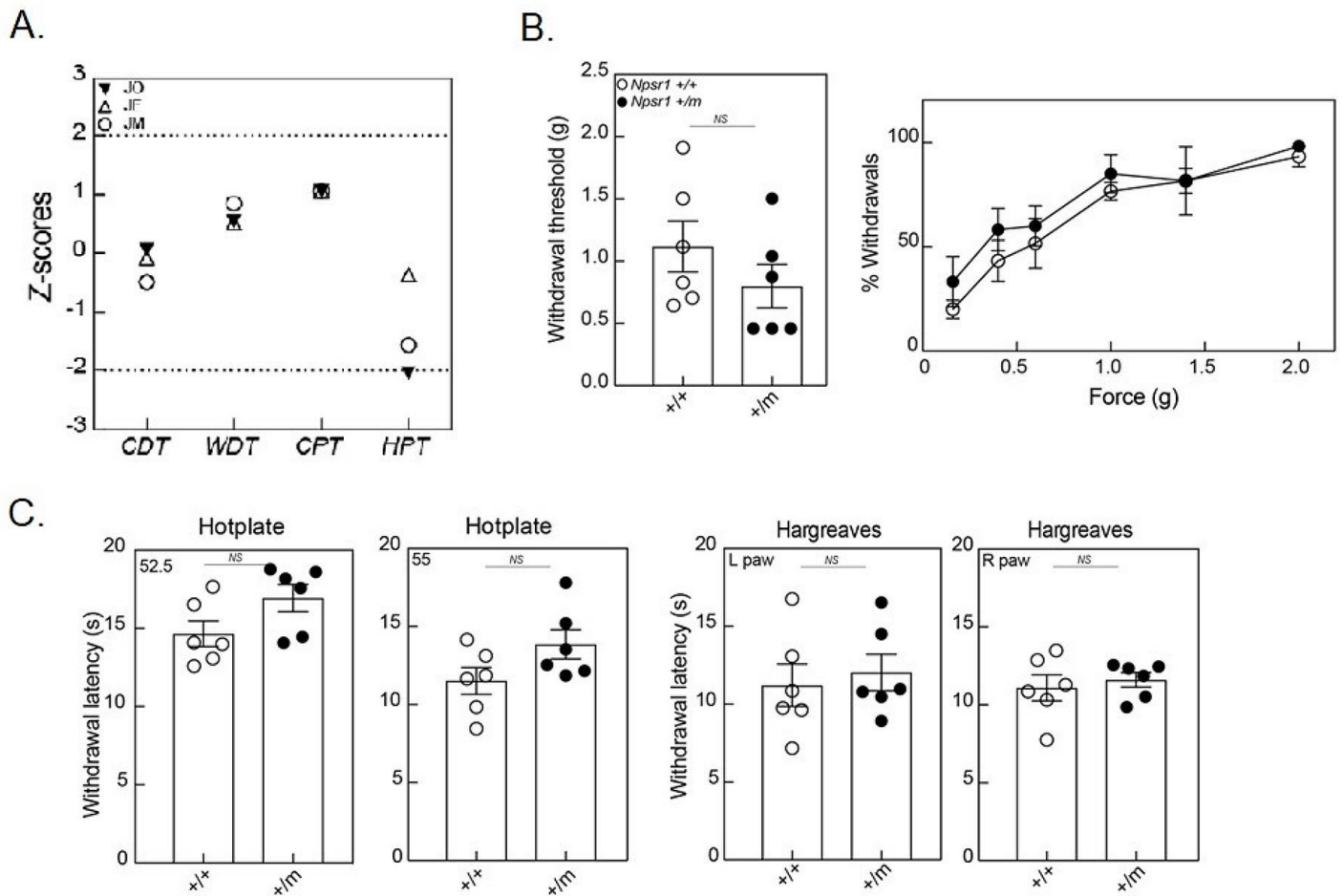
Figure 57: Potential increased pain thresholds in FNSS humans and mice
Unpublished evidence on FNSS provided by Professor Louis Ptáček at the University of California San Francisco. **A)** Thermal pain and discrimination scores (hot and cold) in human FNSS carrying the NSPRF-Y206H mutation, suggesting that FNSS have potential increased cold pain thresholds. **B)** Exploration of mechanical withdrawal thresholds in FNSS mice carrying the NSPRF-Y206H mutation. **C)** Investigation of thermal pain thresholds. There were no significant differences observed. NS = not significant at $p < 0.05$.

In addition to investigating the susceptibility of FNSS mice to migraine-related phenotypes, we aimed to further explore potential underlying mechanisms of any observed changes. One such mechanism may be through alterations in metabolic function and oxidative stress. For example, sleep deprivation leads to oxidative stress (Atrooz & Salim, 2020; Everson et al., 2005; Khadrawy et al., 2011; Villafuerte et al., 2015) defined as disturbance in the balance between the production of reactive oxygen species (free radicals) and antioxidants (Betteridge, 2000) as well as alterations in key metabolites mediating energy metabolism including ATP (Dworak et al., 2010) and its

derivate adenosine (Thuraiaiyah et al., 2022). One function of sleep is thought to be the clearance of waste metabolites from the brain (Hladky & Barrand, 2019; Petit et al., 2021; Xie et al., 2013). For example, during wake interstitial space limits CSF influx and waste metabolites accumulate. During sleep, CSF influx capacity is increased by 60% due to increased interstitial space which facilitates the clearance of these waste metabolites (see **Figure 58**).

Alterations in metabolic and mitochondrial pathways are thought to play a role in migraine pathophysiology (Grech et al., 2021). For example, reduced free energy (ATP), and increased metabolic rate has been observed in patients with FHM (Uncini et al., 1995), and neuroimaging studies have revealed higher ADP (Barbiroli et al., 1992), and lower phosphocreatine (PCr) levels in MA patients (Grech et al., 2021). Further observations support this link as CSD has been shown to be a metabolically demanding event with an increase in the lactate/pyruvate ratio (Santos et al., 2012), ATP demand, and reduction in glucose following CSD (Feuerstein et al., 2016; Leao, 1944; Li et al., 2011). Acute sleep deprivation has been shown to lower the electrical threshold for CSD induction, and this can be recovered by supplementing this energy gap with glycogen or lactate to the interstitium (Kilic et al., 2018). This suggests that an imbalance between mitochondrial dysfunction and increased energetic demand may contribute towards migraine pathophysiology and susceptibility.

FNSS are thought to cope under greater homeostatic sleep pressure and sleep more 'deeply' or efficiently in a shorter time frame (He et al., 2009; Hirano et al., 2018). This could result in more effective clearance of waste metabolites and more free energy available to buffer metabolic demand and the impact of sleep deprivation. Predicated on this, we investigated whole brain metabolite concentrations following acute sleep deprivation and under normal conditions in FNSS.
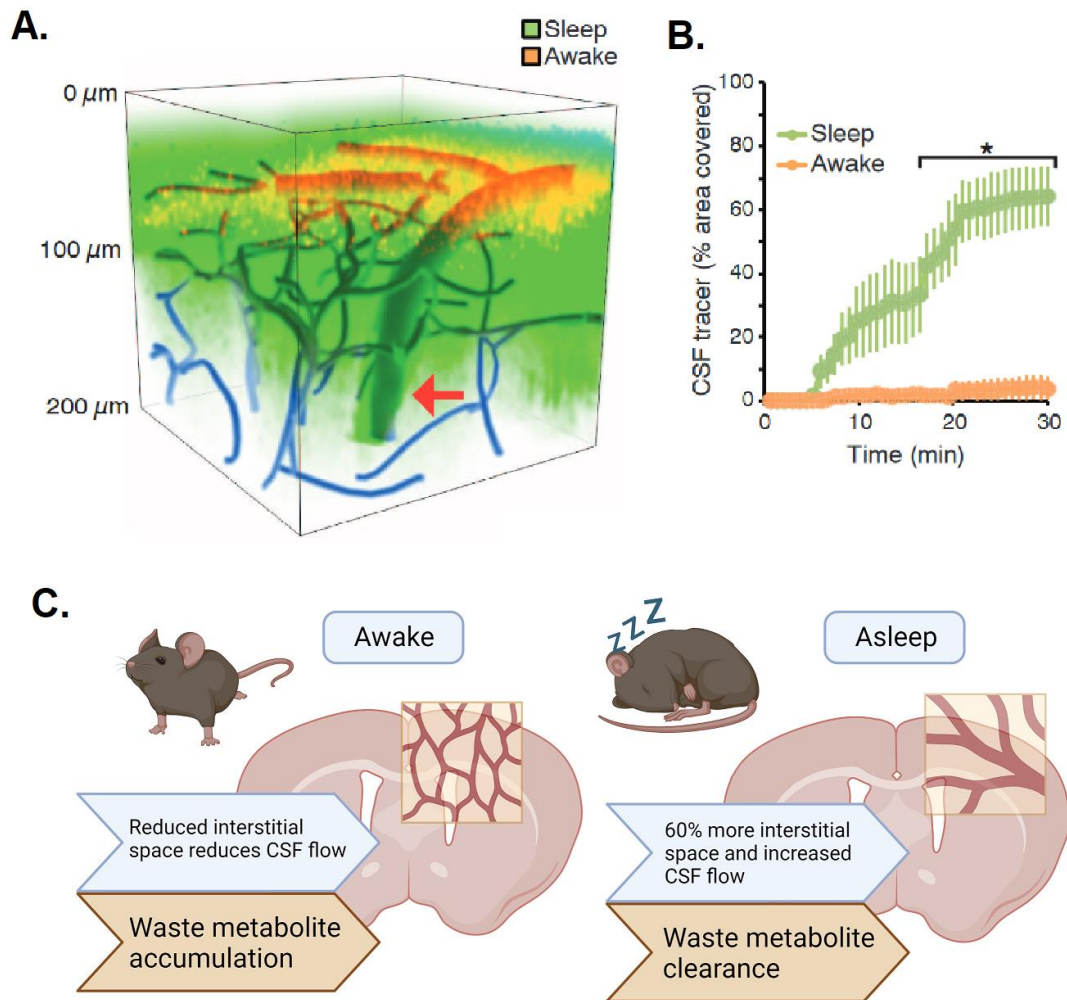
Figure 58: The role of sleep in CSF influx and metabolite clearance
A) Three-dimensional (3D) vectorized reconstruction of the distribution of cerebrospinal fluid (CSF) tracers injected in a sleeping mouse and then again after the mouse was awakened. **B)** Comparison of time-dependent CSF influx in sleep versus awake ($p < 0.01$; t-test). **C)** During wake, reduced interstitial space restricts CSF flow, allowing metabolites to accumulate (Left). In the sleeping brain, aquaporin changes in astrocyte cell volume increase interstitial space to increase CSF access (Right) augmenting metabolite clearance. Adapted from Xie, L., Kang, H., Xu, Q., Chen, M. J., Liao, Y., Thiyagarajan, M., ... & Nedergaard, M. (2013). Sleep drives metabolite clearance from the adult brain. *Science,* 342(6156), 373-377. Reprinted with permission from AAAS.

7.1.1 Aims and hypotheses:

The aims of this chapter were threefold: Firstly, to confirm and validate the use of the *h*DEC2-P384R transgenic mouse line as a model of FNSS; second, to assess the impact of the FNSS mutation on pain and specific migraine-related phenotypes, and finally, to investigate potential mechanisms contributing to the above.

We hypothesise given anecdotal reports of increased pain thresholds that *i*) at baseline and in response to clinical migraine triggers (acute sleep deprivation, NTG), FNSS will have decreased susceptibility to reduced cephalic and extracephalic mechanical withdrawal thresholds compared to WT littermates; *ii*) FNSS will show a reduced susceptibility to CSD events and photophobia*;* and lastly *iii*) this will be accompanied by significant alterations in key metabolites including those mediating energy supply (ATP, ADP, adenosine, and oxidative stress (alanine aminotransferase (ATL); Nicotinamide adenine dinucleotide (NAD+), Nicotinamide (NAM)).

7.2 Methods

To explore whether the human FNSS mutation could impact migraine susceptibility we conducted migraine and sleep readouts in *h*DEC2-P384R, *h*DEC2-WT and WT littermate mice (see **Figure 59** for an overview). The order of testing between behavioural tests was counterbalanced to prevent order effects. Across behavioural studies all measures were assessed at the same circadian time unless otherwise stated.
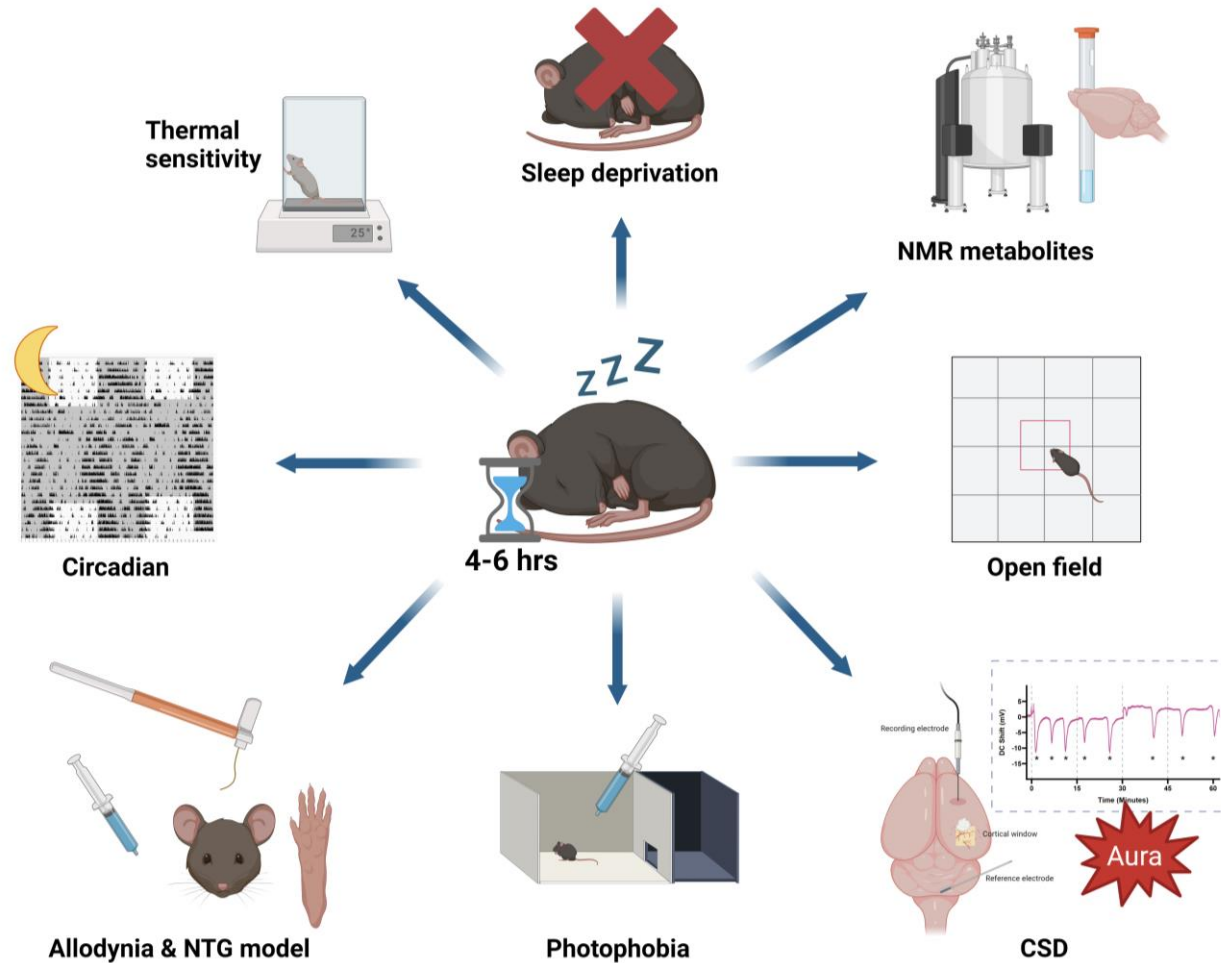
Figure 59: Sleep and migraine-phenotype methodology in FNSS
Schematic showing the range of behavioural and electrophysiological methods used to measure sleep (circadian locomotor activity) and migraine-related phenotypes (CSD, thermal and mechanical allodynia, photophobia, sleep deprivation), and metabolites (NMR) in familial natural short sleeper (FNSS) transgenic mice carrying the P384R mutation in *h*DEC2. Separate cohorts of mice were used to assess CSD, circadian activity, and photophobia. Thermal and mechanical sensitivity assessments were conducted in the same cohort. Sleep deprivation and tissue collection for NMR were also conducted in a separate cohort. CSD = cortical spreading depression, NTG = nitroglycerin, NMR = nuclear magnetic resonance spectroscopy.

7.2.1 Circadian and Sleep Phenotyping in *h*DEC2-P384R and *h*DEC2-WT Mice

Given that the inclusion of a human transgene randomly inserted into the mouse genome could affect behavioural and sleep phenotypes (see section 2.2.1), the *h*DEC2-WT transgenic line was generated by Professor Louis Ptáček's laboratory as a genetic "dosage" control. We conducted a preliminary study to confirm that there were no differences in sleep and circadian measures in the *h*DEC2-WT strain compared to WT littermates. Moreover, this study enabled us to confirm in-house the previously reported short sleep phenotype in *h*DEC2-P384R mice (He et al., 2009).

Whilst performing EEG in these mice would be the gold-standard for determining sleep architecture, unpublished data shared by Dr Winsky-Sommerer's laboratory at the University of Surrey demonstrated that overall sleep-wake activity patterns are equivalent collected via either EEG or passive infrared sensors, suggesting the validity of this technique (see **Figure 60**).
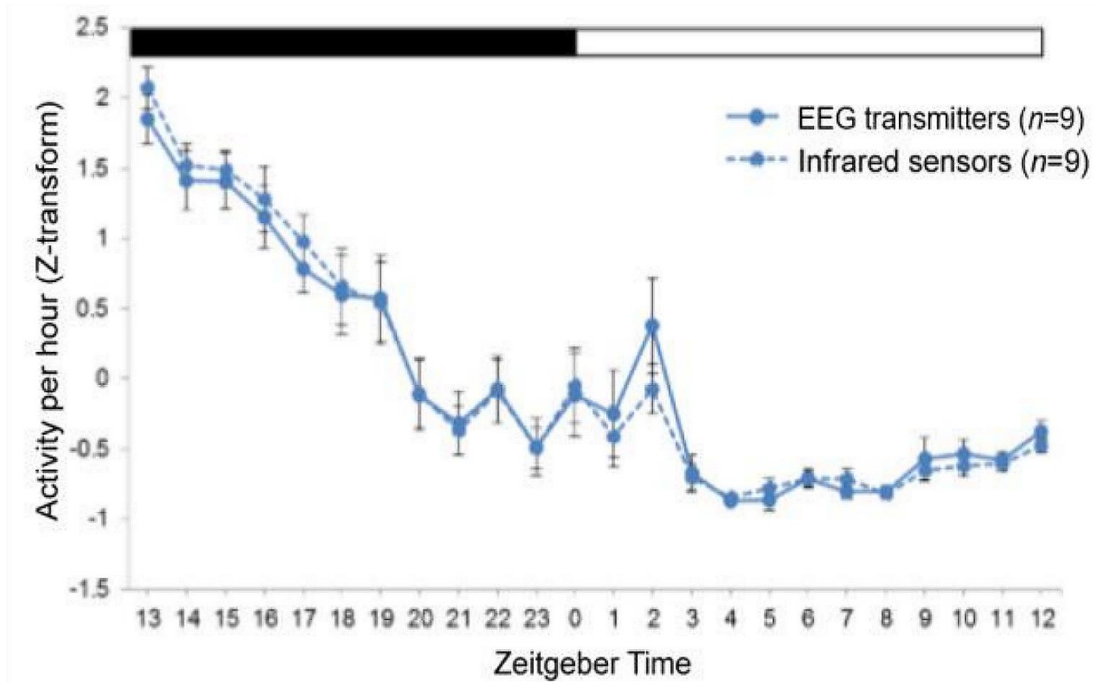
Figure 60: Comparison of EEG and infrared recording
Comparison of sleep/wake activity patterns collected by EEG recording and passive infrared sensors (PIR). Unpublished data shared by Dr Winsky-Sommerer's laboratory at the University of Surrey. Data from both PIR and EEG/EMG recordings underwent a Z-score transformation with resulting values presented as arbitrary units to allow for direct comparison.

34 mice (10 hDEC2-P384R, 12 hDEC2-WT, 12 pooled WT littermates) were included in this experiment. WT littermates from each strain (n = 6) were pooled as we established no significant group differences (all p > 0.05). Mice were placed in a circadian cabinet which measures activity using passive infrared sensors as described in section 2.5 under a normal light-dark (LD) cycle for one week, followed by one week of constant darkness (DD) and one week of normal LD conditions. Other mutations such as that causing FASP, which result in a shortened τ, are not apparent until mice are placed under DD conditions in which endogenous period length can be uncovered (Xu et al., 2005), hence we investigated our FNSS transgenic lines under these different lighting conditions. This also enabled us to confirm whether hDEC2-P384R mice exhibit a sleep rather than a circadian phenotype. Data were assessed by one-way ANOVA with strain (hDEC2-P384R, hDEC2-WT, WT littermates) as the between-

subjects independent variables and the sleep and circadian measures (activity onset and offset (hours), sleep (%), activity period (α), activity counts per minute, period (hours), bout length (minutes), minimum activity (hours), maximum activity (hours), counts per bout, bouts per day) as separate dependent variables. Post-hoc comparisons were conducted where appropriate using t-tests with the Bonferroni correction applied.

7.2.2 Thermal Sensitivity

To determine baseline thermal sensitivity, a separate cohort of hDEC2-P384R (n = 12), hDEC2-WT (n = 12) and WT littermate mice (n = 12 per strain) underwent the hot plate test as described in section 2.6.4. Statistical significance was assessed for both the orofacial and hind paw region by 2-way ANOVA with strain (hDEC2-P384R/hDEC2-WT) and genotype (hemizygous/WT) as the between-subjects independent variables and latency (in seconds) to display a nociceptive response as the dependent variable. Post-hoc Student's t-tests with the Bonferroni correction for multiple comparisons were used to interpret significant main effects and interactions where appropriate.

7.2.3 Orofacial and Hind paw sensitivity

To establish if there were any differences in baseline orofacial and hind paw mechanical withdrawal thresholds these were measured in hDEC2-P384R, hDEC2-WT and WT littermate mice (n = 12 per genotype, the same cohort used to assess thermal sensitivity above) using the von Frey assay as described in section 2.6.1. Thresholds were measured at one circadian time-point: ZT6. Statistical significance was assessed for both the orofacial and hind paw region by 2-way ANOVA with strain (hDEC2-P384R/hDEC2-WT) and genotype (hemizygous/WT) as the between-subjects independent variables and mechanical withdrawal threshold as the

dependent variable. Post-hoc Student's *t*-tests with the Bonferroni correction for multiple comparisons were used to interpret significant main effects and interactions where appropriate.

7.2.4 CSD

To measure phenotypes specific to MA, we conducted CSD electrophysiology in *h*DEC2-P384R ($n = 8$) and *h*DEC2-WT ($n = 8$) transgenic mice as well as WT littermate controls ($n = 8$ per strain) as described in section 2.3.1. To assess differences in the number of CSD events between FNSS and WT littermates, unpaired Student's *t*-tests were conducted between genotypes where appropriate.

7.2.5 NTG-evoked sensitisation

Previous studies have shown that transgenic mice carrying mutations for FASP result in increased sensitivity to the clinical migraine trigger NTG and increased CSD susceptibility (Brennan et al., 2013; Strother, 2020). To assess if FNSS transgenic mice demonstrated any alterations in response to NTG, mice from both transgenic lines and WT littermates ($n = 13/19$ *h*DEC2-P384R and WT littermates, respectively; $n = 11/11$ *h*DEC2-WT and WT littermates, respectively) were counterbalanced to receive 10 mg/kg NTG or vehicle i.p, and orofacial and hind-paw thresholds were assessed two hours post administration as described previously in section 2.6.1. Statistical significance was assessed for both the orofacial and hind paw region by 3-way ANOVA with strain (*h*DEC2-P384R/*h*DEC2-WT), genotype (hemizygous/WT), and drug (NTG/vehicle) as the between-subjects independent variables and mechanical withdrawal threshold as the dependent variable. Post-hoc Student's *t*-tests with the Bonferroni correction for multiple comparisons were used to interpret significant main effects and interactions where appropriate.

7.2.6 Acute Sleep Deprivation

In previous chapters (**chapters 5-6**) we reported that acute sleep deprivation results in a significant reduction of mechanical withdrawal thresholds but has no impact on thermal sensitivity. We used the same protocol to establish whether FNSS exhibit cephalic and extracephalic mechanical and extracephalic thermal allodynia following sleep deprivation. In brief, mechanical, and thermal withdrawal thresholds were measured before and after (ZT6) six hours of acute sleep deprivation in $hDEC2$-P384R mice ($n = 19$, 10 = 6hrSD, 9 = NoSD) and WT littermates ($n = 11$, 4 = 6hrSD, 7 = NoSD). Statistical significance was assessed for both the orofacial and hind paw region for both strains ($hDEC2$-WT and $hDEC2$-P384R) by 2-way ANOVA with session (baseline/post deprivation) and condition (6hrSD or NoSD) as the between-subjects independent variables and mechanical withdrawal threshold as the dependent variable. Tukey's Honestly Significant Difference (HSD) test was used to interpret interactions.

7.2.7 Photophobia

Photophobia is a common symptom experienced during migraine attacks (Goadsby & Holland, 2019). We sought to test a readout of photophobia in $hDEC2$-P384R mice ($n = 18$) and WT littermates ($n = 16$) using the light-dark box assay (see section 2.6.2) two hours post administration of either 10 mg/kg NTG ($n = 9/8$ respectively) or vehicle i.p ($n = 9/8$, respectively). Statistical significance was determined using 2-way ANOVA with genotype (hemizygous/WT) and drug (NTG/Vehicle) as independent variables and photophobia measures as the dependent variables. Post-hoc Student's t-tests with Bonferroni correction for multiple comparisons were used to interpret significant main effects and interactions where appropriate.

7.2.8 Anxiety-like behaviour

As increased orexin has been linked to anxiety in humans and mice, we aimed to assess potential anxiety-like behaviours in FNSS (Lungwitz et al., 2012; Suzuki et al., 2005). To this end, we used the open field arena in hDEC2-P384R mice (n = 12) and WT littermates (n = 12; the same cohort used for basal sensory testing). See section 2.6.3 for detailed methods. To assess initial anxiety-like responses, mice were not habituated to the arena as habituation decreases anxiety-like behaviour (Seibenhener & Wooten, 2015). The measures investigated included: gross movements around the arena, time spent in zone 2 (seconds), number of zone 2 entries, distance travelled in zone 1 (inches). Statistical significance was determined using unpaired Student's t-tests between genotypes.

7.2.9 Metabolites

To explore potential underlying mechanisms, we investigated whether FNSS display alterations in neural metabolites. Whole brains were taken from hDEC2-P384R and WT littermate experimental cohorts above where available (n = 12 hDEC2-P384R and n = 21 WT littermates). We chose to investigate metabolites in the whole brain as studies investigating metabolite changes following sleep deprivation have not generated specific hypotheses regarding metabolites in particular brain regions, but rather an overall clearance of metabolites. A subset of these mice had been sleep deprived for six hours before tissue collection (n = 6 hDEC2-P384R, and n = 9 WT littermates), whereas the remainder were non-sleep-deprived controls. Whole brains were snap frozen in liquid nitrogen at ZT8, two hours following the usual timing of sensory testing in behavioural experiments and processed for NMR spectroscopy as described in section 2.7.6. As we were primarily interested in the difference between genotypes, statistical significance was assessed by unpaired t-tests with the Bonferroni

correction for multiple comparisons applied between hDEC2-P384R mice and WT littermates with metabolite concentrations (μM) as the dependent variable for both sleep deprivation and control conditions.

7.2.10 Prepro-orexin mRNA

Lastly, we sought to confirm previous work in hDEC2-P384R mice which demonstrated significantly increased prepro-orexin mRNA expression at ZT1 in hypothalamic tissue relative to WT littermates (Hirano et al., 2018). However, this study took the samples at the very beginning of the light phase when WT mice are typically asleep. Prepro-orexin (see section 1.7.3) exhibits significant diurnal variation in WT mice, with expression being maximal during the dark phase at ZT20 (Ventzke et al., 2019). What remains unknown is whether hDEC2-P384R mice also show increased prepro-orexin later in the circadian cycle at the time of behavioural testing post sleep deprivation (ZT8). Thus, we sought to explore prepro-orexin mRNA levels using qPCR in hypothalamic-enriched tissue. We conducted this in hDEC2-P384R mice (n = 17) and WT littermates (n = 11) following either acute sleep deprivation (n = 9/4) or no sleep deprivation (n = 8/7) with tissue taken from previous experimental cohorts. In brief, hypothalamic-enriched tissue was taken by removing the inferior section of the whole brain (in the coronal plane) and snap-freezing them in liquid nitrogen. They were analysed by qPCR for prepro-orexin as described in the methods section 2.7.5. Statistical significance was assessed by 2-way ANOVA with genotype and condition (sleep deprivation / no sleep deprivation) as the between-subjects independent variables and prepro-orexin mRNA transcription factor levels as the dependent variable. Post-hoc Student's t-tests with Bonferroni correction for multiple comparisons were used to interpret significant main effects and interactions where appropriate.

In all studies, data are reported as mean ± SD, and statistical significance was set at α = 0.05 unless otherwise stated.

7.3 Results

7.3.1 Confirmation of FNSS Phenotype in *h*DEC2-P384R mice

To confirm the short sleep phenotype in FNSS, locomotor activity was assessed during LD and free-running DD conditions (**Figure 61**). Descriptive and inferential statistics are shown in **Table 21.** During LD conditions there was no significant difference in period (τ) between *h*DEC2-P384R, *h*DEC2-WT and WT littermates ($F_{(2,31)}$ = 2.38; p = 0.109). However, during DD conditions, endogenous τ was significantly shorter in *h*DEC2-P384R mice compared to *h*DEC2-WT (23.7hrs ± 0.11 vs 23.8hrs ± 0.10; t_{19} = 2.83; p = 0.032; **Figure 62A**), indicating a subtle circadian phenotype in FNSS mice. However, τ values were similar to previous reports (*h*DEC2-P384R: 24.04 ± 0.02hr LD; 23.78 ± 0.09 DD; WT: 24.02 ± 0.02 LD; 23.68 ± 0.06 DD; He et al., 2009).

During LD conditions, there was a significant difference in sleep amount during the dark phase ($F_{(1,32)}$ = 2.91; p = 0.014; **Figure 63C**) but not the light phase ($F_{(1,32)}$ = 1.10; p = 0.359). *h*DEC2-P384R mice slept significantly less than WT littermates (34.0% ± 12.10 vs 50.10% ± 19.40; t_{20} = 2.03, p = 0.046) but not *h*DEC2-WT (45.70% ± 14.90; $t_{20.6}$ = 0.63, p = 0.536). Similarly, there was no difference between transgenic lines during the light phase in activity counts per minute ($F_{(2,31)}$ = 0.27; p = 0.769). However, in the dark phase, *h*DEC2-P384R mice displayed significantly more activity counts than *h*DEC2-WT (5.69 ± 1.58 vs 4.37 ± 1.77; $t_{18.5}$ = -2.76; p = 0.008) and WT littermates (3.89 ± 1.44; $t_{19.9}$ = -2.81; p = 0.013; **Figure 63A**).

Contrary to previous reports we reported no significant difference in the total duration of the activity period (α) between hDEC2-P384R, hDEC2-WT and WT littermates ($F(1,31)$ = 1.72; p = 0.195) under LD conditions. There was a significant difference in activity offset relative to ZT0 ($F(1,32)$ = 7.17; p = 0.013). Activity offset was approximately 2.1 hours later in DEC2-P384R mice than DEC2-WT (8.85 ± 2.71 vs 6.75 ± 0.68; t_{13} = -2.57, p = 0.024) or WT mice (6.72 ± 1.35; $t_{15.2}$ = -2.44; p = 0.027). This is in line with the activity pattern seen in humans carrying the P384R mutation in hDEC2; exhibiting the same onset time to conventional sleepers but waking earlier by 2-3 hours (see **Figure 46B**).

There was no significant difference in bout length (minutes; $F(1,32)$ = 0.16; p = 0.852), counts per bout ($F(1,32)$ = 0.23; p = 0.796), bouts per day ($F(1,32)$ = 0.48; p = 0.624), activity onset ($F(1,32)$ = 0.50; p = 0.614), or maximum or minimum activity ($F(1,32)$ = 3.20; p = 0.056) across transgenic lines.

Table 23: Descriptive and inferential statistics for sleep phenotyping in FNSS

	WT (n = 12)	hDEC2-WT (n = 12)	hDEC2-P384R (n = 10)	$F_{2,31}$	p-value
Period in LD (hours)	23.8 (0.21)	24.0 (0.08)	23.9 (0.11)	2.38	0.109
Period in DD (hours)	23.8 (0.13)	23.8 (0.10)	23.7 (0.11)	3.59	0.040*
Activity counts (per minute)					
Light	2.45 (0.78)	2.56 (0.73)	2.33 (6.11)	0.27	0.769
Dark	3.89 (1.44)	4.37 (1.77)	5.69 (1.58)	3.60	0.039*
Activity (LD)					
Activity period (a)	12.7 (2.42)	14.1 (3.05)	15.1 (3.63)	1.72	0.195
Activity onset (hours)	17.6 (1.84)	17.6 (1.31)	16.8 (2.85)	0.50	0.614
Activity offset (hours)	6.72 (1.35)	6.75 (0.68)	8.85 (2.71)	7.17	0.013*
Max Activity (hours)	9.90 (2.14)	9.43 (3.55)	8.18 (1.55)	1.08	0 .354
Min Activity (hours)	0.75 (1.00)	1.31 (1.02)	0.29 (0.45)	3.20	0 .056
Sleep (%)					
Light	69.3 (7.52)	71.4 (5.04)	67.4 (6.07)	1.10	0.359
Dark	50.1 (19.4)	45.7 (14.9)	34.0 (12.1)	2.91	0.014*
Bouts (LD)					
Bout length (minutes)	27.1 (11.5)	25.8 (11.7)	24.6 (7.82)	0.16	0.852
Counts per bout	197 (110)	175 (75.5)	203 (115)	0.23	0.796
Bouts/day	21.7 (6.17)	23.5 (8.54)	24.4 (5.64)	0.48	0.624

Data are presented as mean (SD); p-values computed using one-way ANOVA. LD: 12-hour light, DD: constant darkness. 12-hour dark light cycle, ZT = Zeitgeber time.
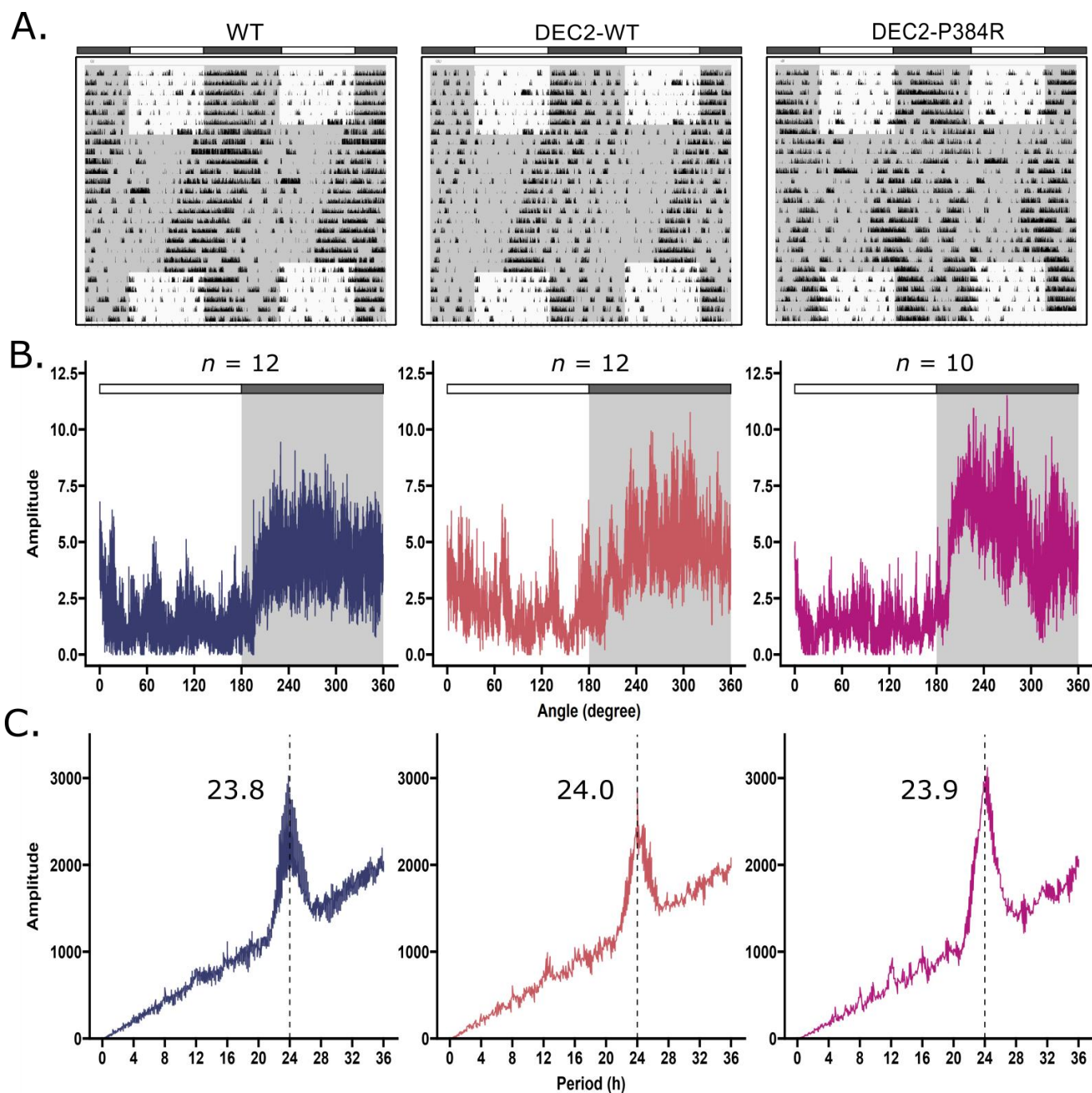
Figure 61: Circadian locomotor activity in FNSS transgenic mice
A) Circadian locomotor activity for FNSS mice (*h*DEC2-P384R; *n* = 10), *h*DEC2-WT (*n* = 12) and wild-type (WT; *n* = 12) littermate mice recorded by PIR during two weeks of normal light-dark (LD) conditions, two weeks of constant darkness (DD), and two weeks of normal LD conditions. Data are double plotted for visualisation purposes. **B)** Activity onset analysis with lights on at 07:00 (ZT0) and lights off at 19:00 (ZT12) during normal LD conditions. **C)** Periodograms of locomotor activity during days 1-14 (LD conditions), superimposed with the average period length in hours.
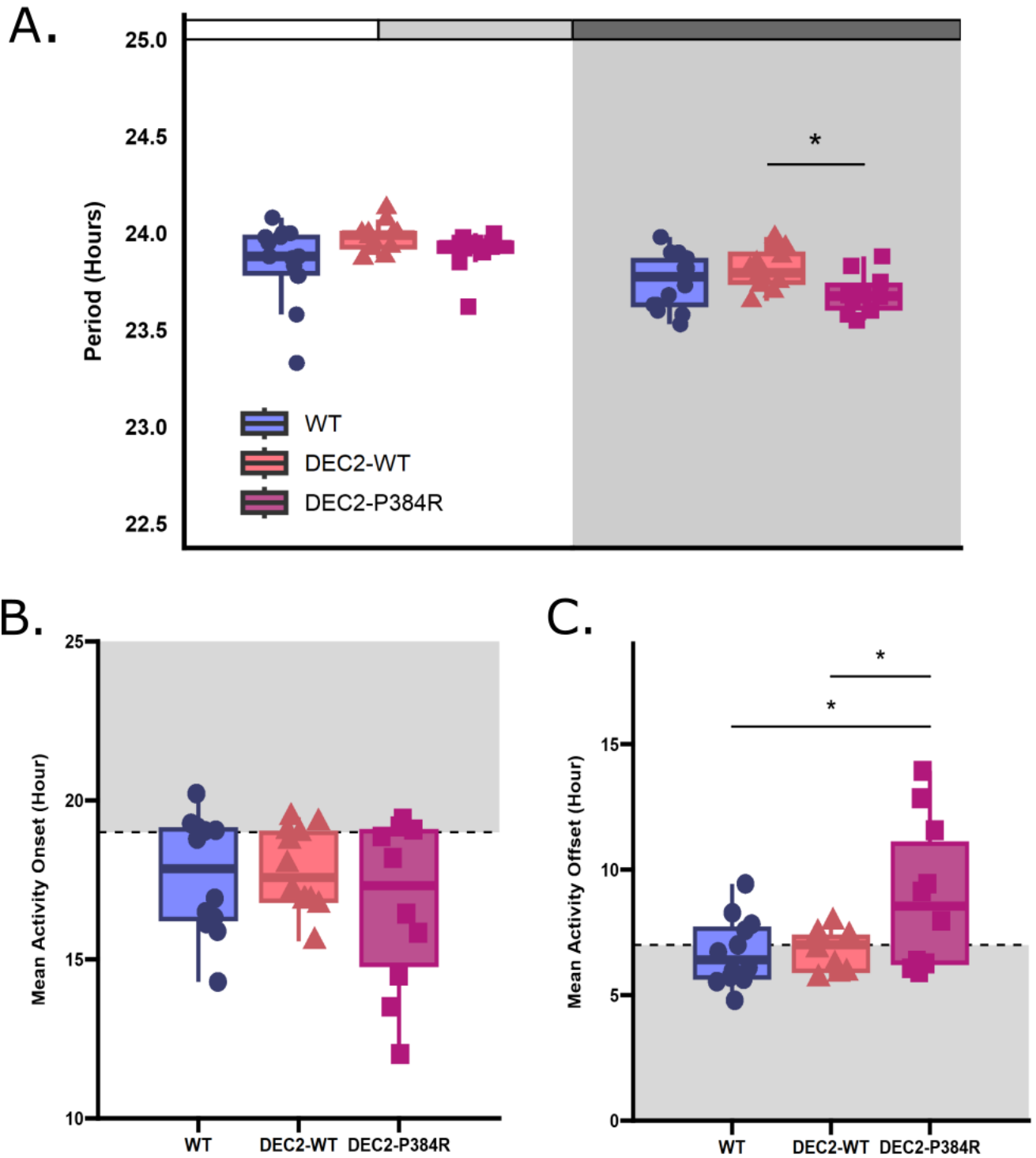
Figure 62: Period and activity onset and offset in *h*DEC2-P384R mice

A) There was no difference in τ between strains ($F_{(2,31)}$ = 2.38; p = 0.109) during LD conditions, but there was a difference in endogenous τ under DD conditions ($F_{2,31}$ = 3.59; p = 0.040). τ was significantly shorter in *h*DEC2-P384R compared to *h*DEC2-WT mice (t_{19} = 2.83; p = 0.032). **B)** There were no differences in activity onset between genotypes ($F_{(1,32)}$ = 10.5; p = 0.614). The dashed line represents "lights off" (ZT12). **C)** *h*DEC2-P384R mice had a significantly later activity onset than *h*DEC2-WT (t_{13} = -2.57, p = 0.024) and WT littermates ($t_{15.2}$ = -2.44; p = 0.027). The dashed line represents "lights off" (ZT12). Data represent mean ± SEM. Shapes represent data from individual animals. * = $p < 0.05$ computed using Student's *t*-test with the Bonferroni correction applied.
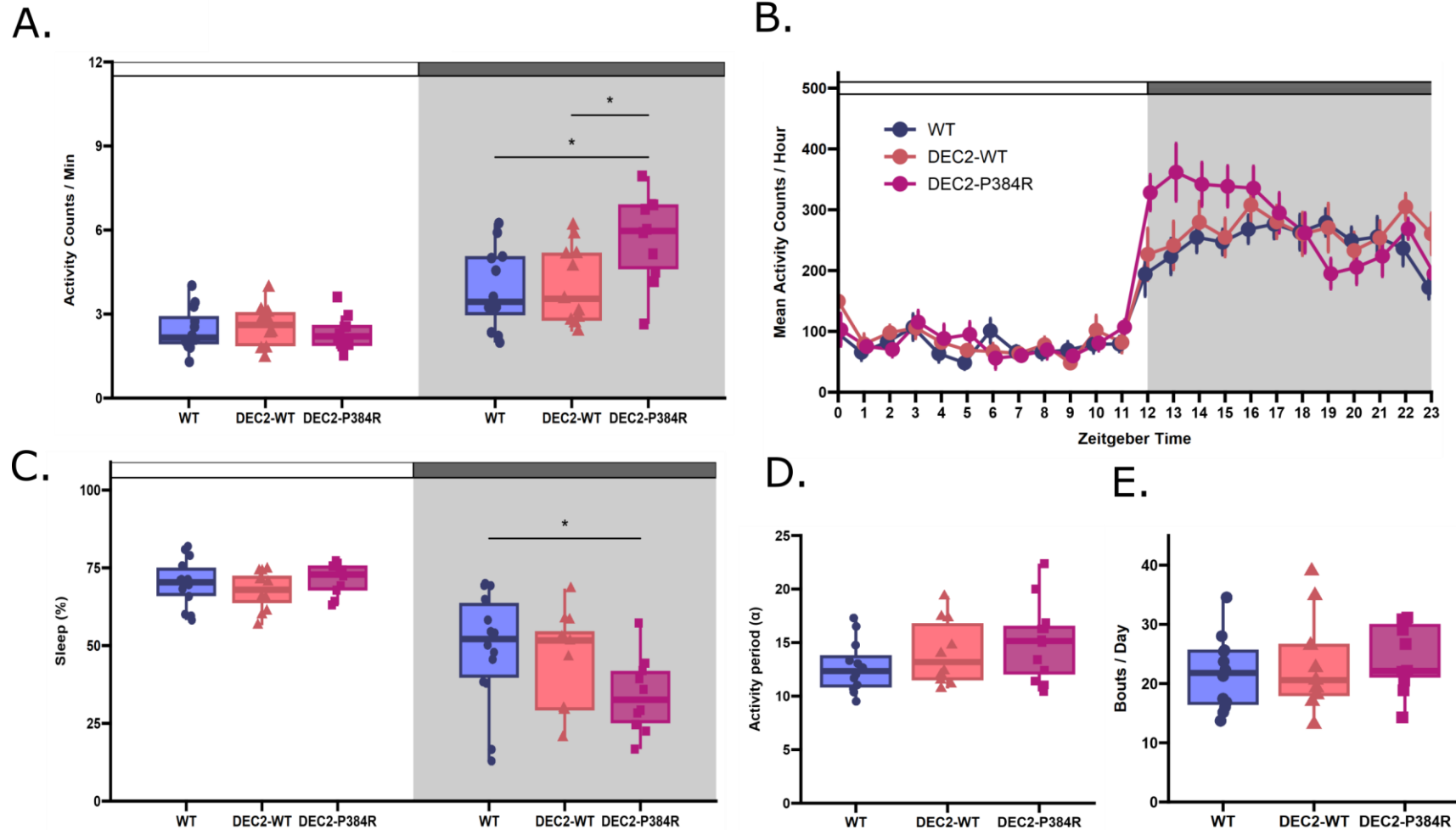
Figure 63: Reduced sleep in the dark phase in FNSS

A) Average activity counts per minute did not vary across strains in the light phase. However, during the dark phase, hDEC2-P384R mice ($n = 10$; squares) were significantly more active than WT ($n = 12$; circles; $t_{19.9} = -2.81$; $p = 0.013$), and hDEC2-WT mice ($n = 12$; triangles; $t_{18.5} = -2.76$; $p = 0.008$). **B)** Mean activity counts per hour over 2 weeks plotted according to Zeitgeber time, showing an increase in activity at the start of the dark phase in hDEC2-P384R mice. **C)** Percent sleep proxy measure calculated from activity patterns. There was no difference in sleep between strains in the light phase ($F(1,32) = 1.10$; $p = 0.359$); however, in the dark phase hDEC2-P384R mice had significantly less sleep compared to WT ($t_{20} = 2.03$, $p = 0.046$). **D)** There was no effect of genotype on activity period ($F(1,32) = 1.72$; $p = 0.195$). **E)** There was no effect of genotype on bouts per day ($F(1,32) = 0.48$; $p = 0.624$). Abbreviations: WT = wild-type. Data represent mean ± SEM. Shapes represent data from individual animals. ** = $p < 0.01$; * = $p < 0.05$ computed using Student's t-test with the Bonferroni correction applied.

269

7.3.2 No Differences in Thermal Sensitivity in FNSS

To assess whether FNSS exhibit alterations in baseline sensory sensitivity, we explored sensitivity to a noxious thermal stimulus using the hotplate test. There was no impact of genotype ($F(1,44) = 0.12$, $p = 0.731$) or strain ($F(1,44) = .99$, $p = 0.326$; **Figure 64**) on latency to display a nociceptive response, suggesting no differences in baseline thermal sensitivity in FNSS.

7.3.3 No Differences in Cephalic or Extracephalic Sensitivity in FNSS

We investigated orofacial and hind paw sensitivity during baseline conditions using the von Frey assay. Two mice from the hDEC2-WT strain were excluded after habituation due to failure to habituate to the testing apparatus (final $n = 11$ hDEC2-WT, $n = 12$ hDEC2-P384R, $n = 11/12$ WT littermates respectively). Neither strain (hDEC2-WT vs hDEC2-P384R; $F(1,50) = 0.14$, $p = 0.710$, $\eta^2_G = 0.02$) nor genotype (hemi vs WT; $F(1,50) = 0.32$, $p = 0.572$, $\eta^2_G = 0.01$) significantly impacted orofacial (**Figure 65A**) or hind paw mechanical withdrawal thresholds (Strain: $F(1,42) = 0.23$, $p = 0.635$, $\eta^2_G = 0.01$; Genotype: $F(1,42) = 0.52$, $p = 0.476$, $\eta^2_G = 0.01$; **Figure 65B**), suggesting no differences in baseline mechanical sensitivity in FNSS.
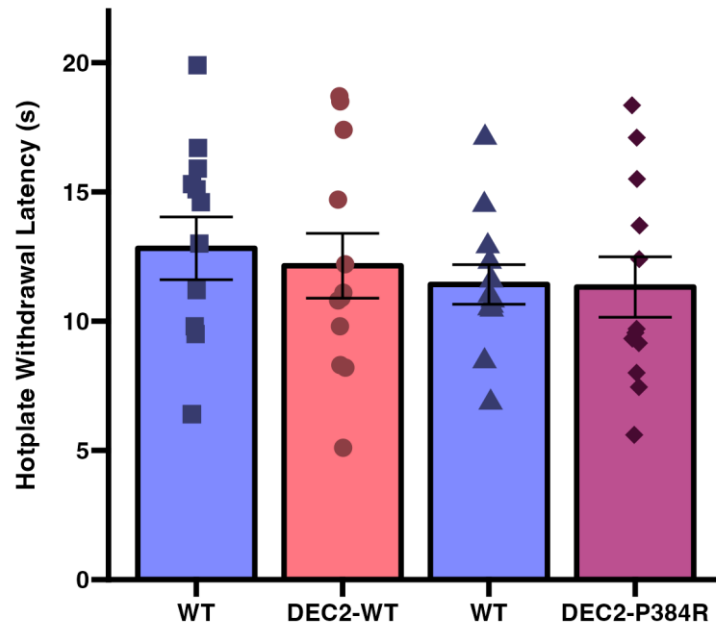
Figure 64: No difference in thermal sensitivity in transgenic FNSS mice
No significant difference in hotplate withdrawal latencies in hDEC2-P384R (n = 12) or hDEC2-WT (n = 11) mice and corresponding wild-type (WT) littermates (n = 12/12 respectively; $F_{(1,44)}$ = .99, p = 0.326; Genotype (hemizygous vs WT; $F_{(1,44)}$ = 0.12, p = 0.731). Abbreviations: WT = wild type. Data represent mean ± SEM. Shapes represent data from individual animals.
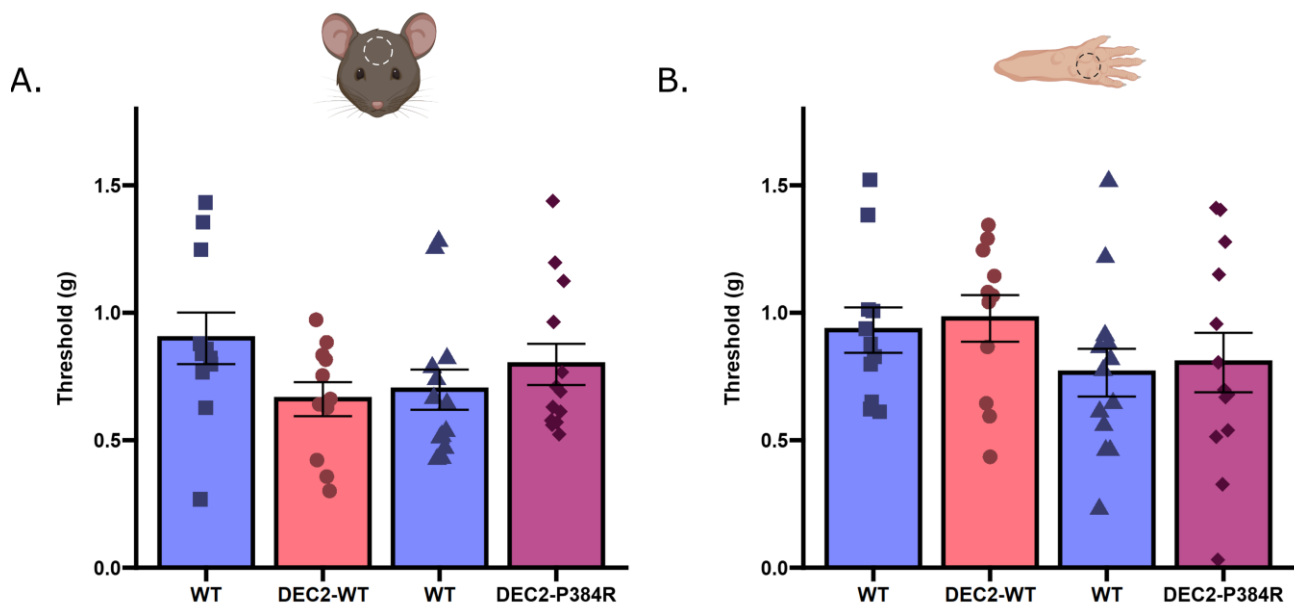
Figure 65: Basal mechanical withdrawal thresholds in FNSS
A) No significant difference in periorbital mechanical withdrawal thresholds measured by the von Frey assay in hDEC2-P384R (n = 12) or hDEC2-WT (n = 11) mice and corresponding wild-type (WT) littermates (n = 12/11 respectively; Strain: $F_{(1,50)}$ = 0.14, p = 0.710; Genotype (hemizygous vs WT; $F_{(1,50)}$ = 0.32, p = 0.572). **B**) No significant difference in hind-paw mechanical withdrawal thresholds in hDEC2-P384R (n = 12) or hDEC2-WT (n = 11) mice and corresponding WT littermates (n = 12/11 respectively; Strain: $F_{(1,42)}$ = 0.23, p = 0.635; Genotype: $F_{(1,42)}$ = 0.52, p = 0.476). Shapes represent data from individual animals. Data represent mean ± SEM, and inferential statistics were computed using 2-way ANOVA where α = 0.05.

7.3.4 No Difference in CSD Susceptibility in FNSS

To explore whether alterations in natural sleep duration affect cortical excitability, CSD events were assessed in *h*DEC2-P384R and *h*DEC2-WT mice and WT littermates. Data from two *h*DEC2-P384R mice was lost due to issues with the recording setup (final n = 6). Data were not normally distributed thus the Mann-Whitney U test was computed and are represented as median (Mdn) and interquartile range (IQR).

*h*DEC2-P384R mice showed no significant difference in the number of CSD events compared to WT littermates (Mdn = 7 [7-7] vs Mdn = 7.5 [3.5-11.5]; U = 27.5, p = 0.689; **Figure 66D**). *h*DEC2-WT mice did not differ in the number of CSDs compared to their WT littermates (7 [6-8] vs 7 [6-8]; U = 37.5, p = 0.562; **Figure 66C**) indicating that natural sleep duration has no impact on migraine-related cortical excitability.
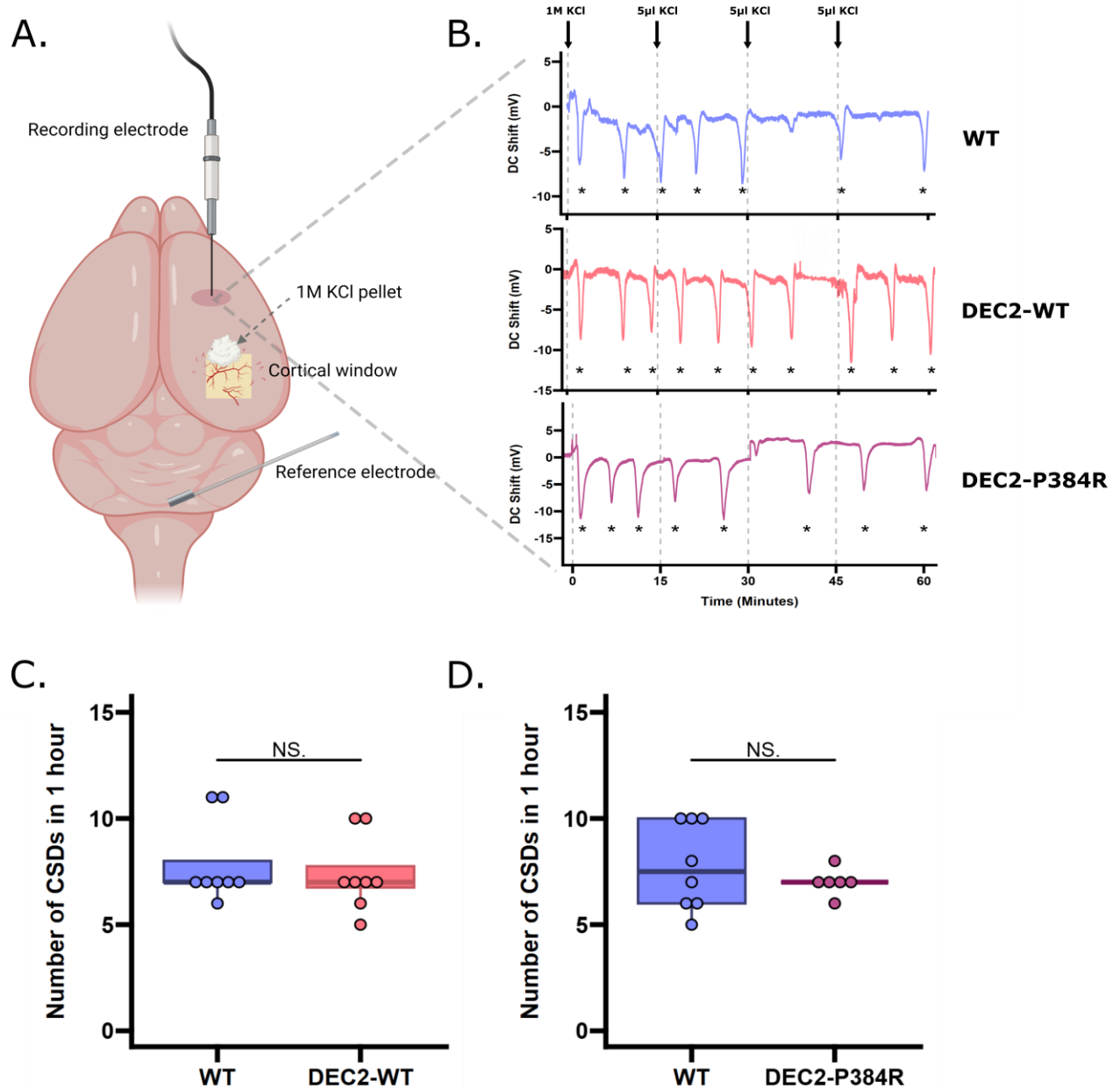
Figure 66: No difference in cortical spreading depression events in FNSS
A) Schematic of the CSD model in an anaesthetised animal. **B)** Representative traces of changes in DC coupled (DC) electrical potential in response to 1M KCl for 1 hour followed by 5 µl applied every 15 minutes (black arrows). Asterixis denote where a CSD event was counted. **C-D)** Both *h*DEC2-WT (*n* = 8/8; *U* = 37.5, *p* = 0.562) and *h*DEC2-P384R (*n* = 6/8; *U* = 27.5, *p* = 0.689) transgenic lines showed no significant difference in the number of CSD events in one hour compared to their respective wild-type (WT) littermates. Data represent median and interquartile range (IQR). Circles represent data points from individual animals. NS. = not significant at *p* < 0.05 computed using the Mann-Whitney *U*-test.

7.3.5 Altered Response to a Clinical Migraine Trigger in FNSS

Periorbital thresholds

To assess whether FNSS are less susceptible to migraine triggers we investigated NTG-evoked periorbital sensitivity in hDEC2-P384R, hDEC2-WT and WT littermates. There was a significant impact of drug on threshold ($F(1,100) = 1.58$, $p = 0.002$, = 0.01). No other main effects were significant. NTG administration significantly reduced periorbital mechanical withdrawal thresholds in mice expressing the hDEC2-WT allele and WT littermates compared to vehicle injection (WT: 0.17g ± 0.15 vs 0.97g ± 0.33; $t_9 = 5.38$, $p = 0.009$; hDEC2-WT 0.40g ± 0.24 to 0.90g ± 0.25; $t_9 = 3.34$, $p < 0.001$; **Figure 67A**). Whilst WT littermates were significantly sensitised by NTG compared to vehicle injection (0.35g ± 0.24 vs 0.78g ± 0.52; $t_{17} = 2.30$, $p = 0.035$; **Figure 67C**), hDEC2-P384R mice were not (0.63g ± 0.58 vs 0.85g ± 0.50; $t_{11} = 0.74$, $p = 0.476$; **Figure 67C**).

Hind paw thresholds

To confirm whether this effect was specific to cephalic allodynia, we next explored NTG-evoked sensitivity in the hind paw. NTG administration did not significantly affect mechanical withdrawal thresholds in the hind paw (all $p > 0.05$; see **Figure 67B,D**).
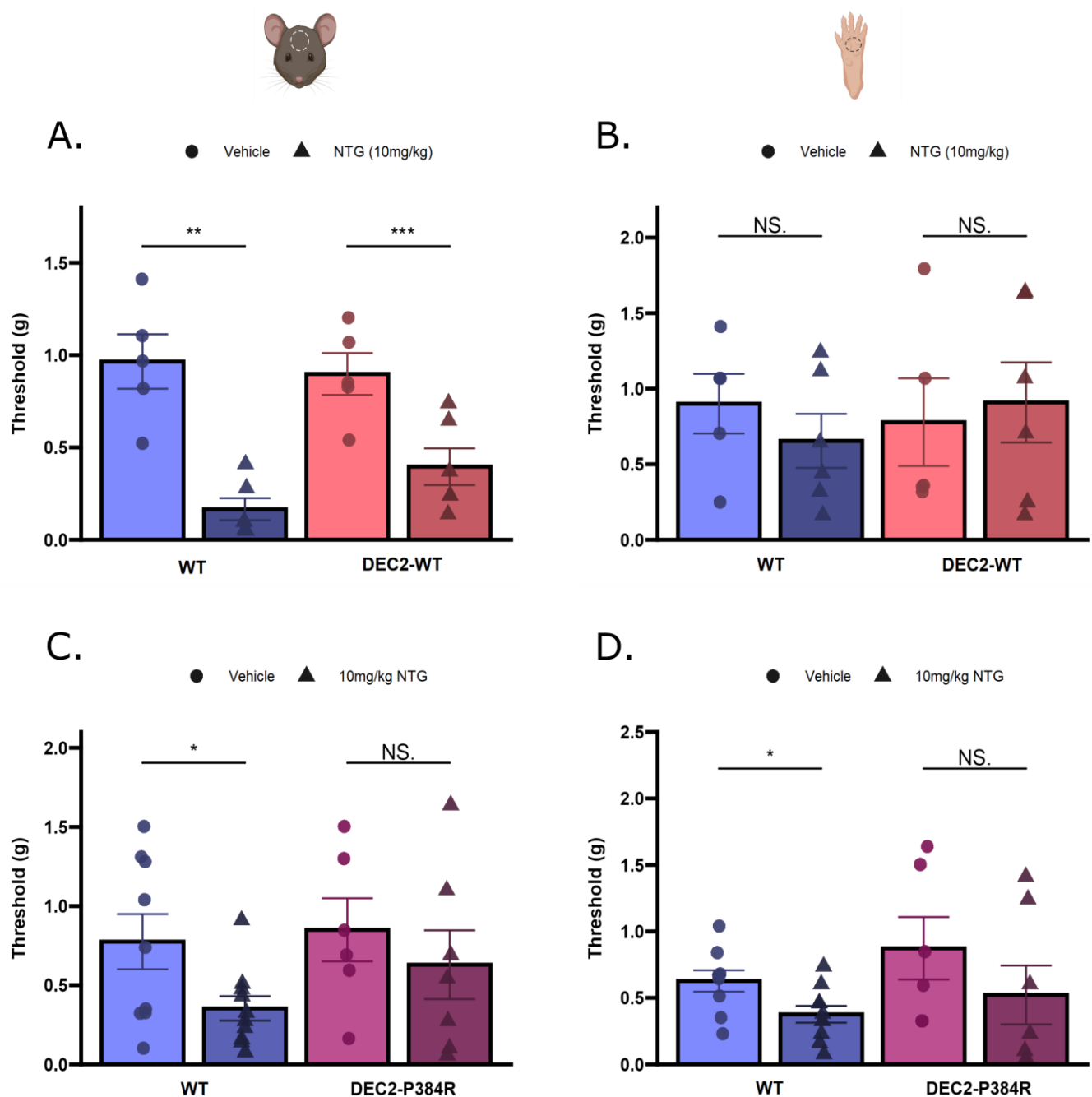
Figure 67: Reduced periorbital allodynia in response to NTG in FNSS mice
A) Both hDEC2-WT and corresponding WT littermates displayed lower periorbital mechanical withdrawal thresholds two hours post NTG (10 mg/kg) administration compared to vehicle ($n = 5/5$; $t_9 = 5.38$; $p < 0.001$; $n = 5/5$; $t_9 = 3.34$; $p = 0.009$). **B)** Neither hDEC2-WT ($n = 5/5$; $t_9 = 0.05$; $p = 0.959$) or WT littermates showed reduced hind paw thresholds with NTG versus vehicle ($n = 4/6$; $t_9 = 0.34$; $p = 0.741$). **C)** hDEC2-P384R mice did not show reduced periorbital mechanical withdrawal thresholds following NTG vs vehicle ($n = 7/6$; $t_{11} = 0.74$; $p = 0.476$), whereas WT littermates did ($n = 10/9$; $t_{17} = 2.30$; $p = .035$). **D)** hDEC2-P384R mice did not show reduced hind paw thresholds following NTG vs vehicle ($n = 7/6$; $t_{11} = 1.09$; $p = 0.301$) but WT littermates did ($n = 10/9$; $t_{17} = 2.47$; $p = 0.024$). Abbreviations: NTG = nitroglycerin; WT = wild-type. Data represent mean ± SEM. Shapes represent data from individual animals. NS. = not significant, * = $p < 0.05$, ** = $p < 0.01$, *** $p < 0.001$ computed using Student's t-test with the Bonferroni correction for multiple comparisons applied.

7.3.6 Acute Sleep Deprivation Induces Periorbital Allodynia in FNSS

<u>Periorbital thresholds</u>

We next sought to explore whether FNSS exhibit the mechanical hypersensitivity previously observed following acute sleep deprivation. Replicating previous chapters, acute sleep deprivation affected orofacial mechanical withdrawal thresholds in WT mice ($F(1,18) = 9.05$, $p = 0.008$, $\eta^2_G = 0.29$). WT sleep deprived mice had significantly lower orofacial mechanical withdrawal thresholds than non-sleep-deprived mice (0.46g ± 0.40 vs 0.96g ± 0.39, $p = 0.007$; **Figure 68A**). Sleep deprivation also had an effect in hDEC2-P384R mice ($F(1,34) = 6.24$, $p = 0.018$, $\eta^2_G = 0.15$) in that sleep deprived mice had significantly lower orofacial mechanical withdrawal thresholds than non-sleep-deprived mice (0.54g ± 0.44 vs 0.92g ± 0.50, $p = 0.018$; **Figure 68C**).

<u>Hind paw thresholds</u>

For both hDEC2-P384R and WTs, acute sleep deprivation did not affect extracephalic mechanical sensitivity (all $p > 0.05$) measured in the hind paw (**Figure 68B-D**).
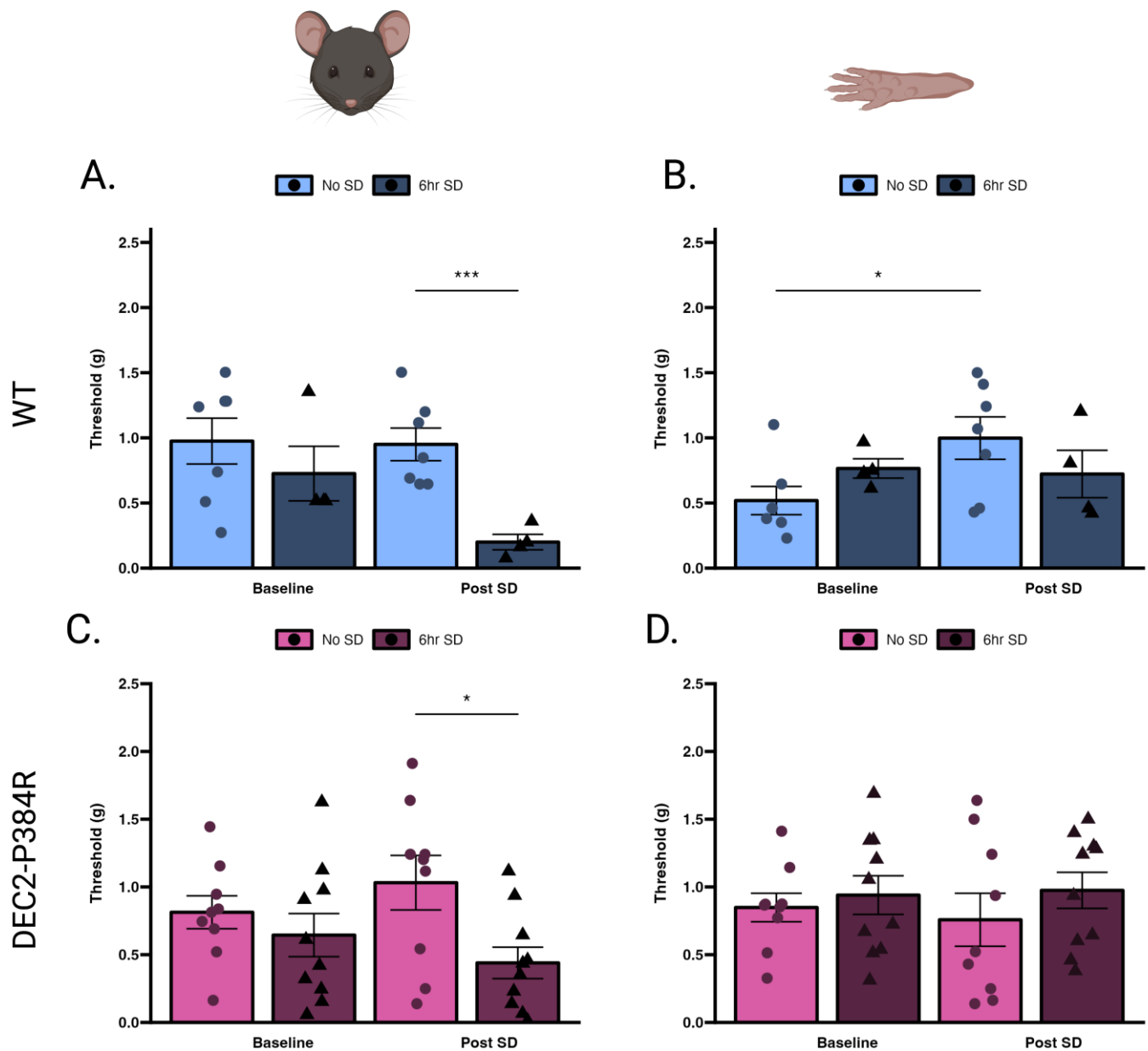
Figure 68: Periorbital allodynia after acute sleep deprivation in FNSS
Acute sleep deprivation (triangles) significantly reduced periorbital mechanical withdrawal thresholds in hDEC2-P384R mice (**C**; n = 10/9, p = 0.018) and WT littermates (**A**; n = 4/7, p = 0.007) compared to non-sleep-deprived controls (circles). Acute sleep deprivation did not significantly reduce hind paw thresholds in hDEC2-P384R mice (**D**; n = 10/9) or WT littermates (n = 4/7; **B**) (both p > 0.05). Data represent mean ± SEM. Shapes represent data from individual animals. *** = p < 0.01, * = p < 0.05 computed using Tukey's HSD.

7.3.7 Thermal Allodynia in Response to Acute Sleep Deprivation in FNSS

As well as investigating mechanical sensitivity following sleep deprivation, we also assessed thermal sensitivity in the hind paw using the hot plate test. Acute sleep deprivation significantly affected thermal sensitivity ($F(1,24) = 8.40$, $p = 0.008$, $\eta^2_G = 0.21$) and there was significant variation in thermal latencies across sessions ($F(1,24) = 7.14$, $p = 0.013$, $\eta^2_G = 0.18$). Sleep-deprived hDEC2-P384R mice displayed a significant reduction in latency to display a noxious response (12.0s ± 1.54 vs 8.89s ± 1.01, $t_{10.4} = 4.44$, $p = 0.001$; **Figure 69**), whereas non-sleep-deprived mice did not (13.5s ± 2.54 vs 12.2s ± 3.04, $t_{11.6} = 0.88$, $p = 0.398$).
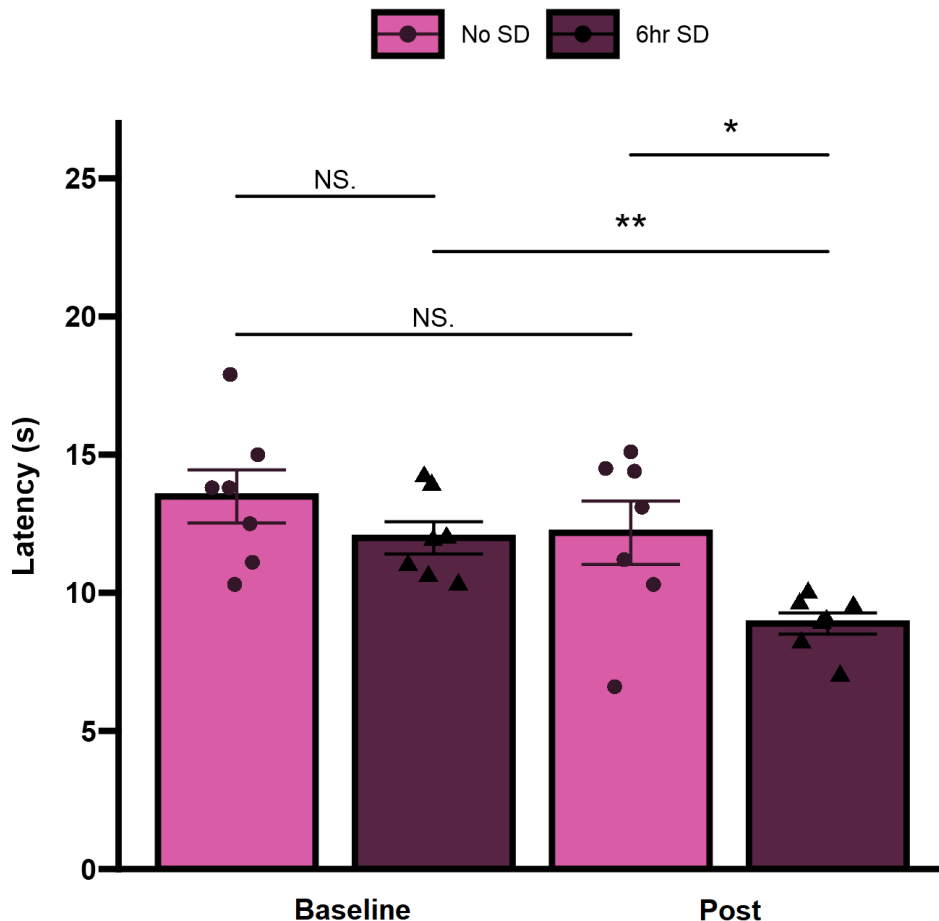
Figure 69: Acute sleep deprivation induces thermal allodynia in FNSS
Acute sleep deprivation in hDEC2-P384R mice results in a reduction of latency (seconds) to respond on the hotplate test (6hrSD; $n = 7$; triangles; $t_{10.4} = 4.44$, $p = 0.001$) versus non-sleep-deprived controls (NoSD; $n = 7$; circles; $t_{11.6} = 0.88$, $p = 0.398$) indicating thermal hyperalgesia. Data represent mean ± SEM. Shapes represent data from individual animals. NS. = not significant, ** = $p < 0.01$, * $p < 0.05$ computed using Student's t-test with the Bonferroni correction for multiple comparisons applied.

7.3.8 Differential Photophobic Response in FNSS

To explore whether FNSS display resistance to a common migraine symptom: photophobia, we tested light sensitivity using the light-dark box in WT and hDEC2-P384R mice two hours post administration of the clinical migraine trigger NTG. There was a significant genotype effect in that WT mice spent significantly less time in the light chamber versus hDEC2-P384R mice (179 ± 45.1 vs 266 ± 86.6 seconds; $F(1,30) = 14.27$, $p < 0.001$; **Figure 70A**). However, NTG administration did not affect this phenotype ($p = 0.296$).

hDEC2-P384R mice had a shorter latency to re-enter the light chamber than WT mice (20.8 ± 9.36 vs 38.8 ± 37.1 seconds; $F(1,30) = 5.07$), $p = 0.032$; **Figure 70C**). There was a significant interaction between drug and genotype in that WT mice given NTG (58 ± 43.6 seconds) had significantly longer latencies than hDEC2-P384R mice given NTG (17.8 ± 9.78) or vehicle (23.9 ± 8.34; $F(1,30) = 7.86$, $p = 0.009$). Drug or genotype had no significant impact on latency to enter the dark chamber for the first time ($F(1,30) = 4.01$, $p = 0.054$; $F(1,30) = 0.26$, $p = 0.616$; **Figure 70B**). hDEC2-P384R mice transitioned between chambers significantly more than WT mice ($F(1,30) = 7.70$, $p = 0.009$; **Figure 70D**), but transitions did not differ between drugs ($p = 0.057$).
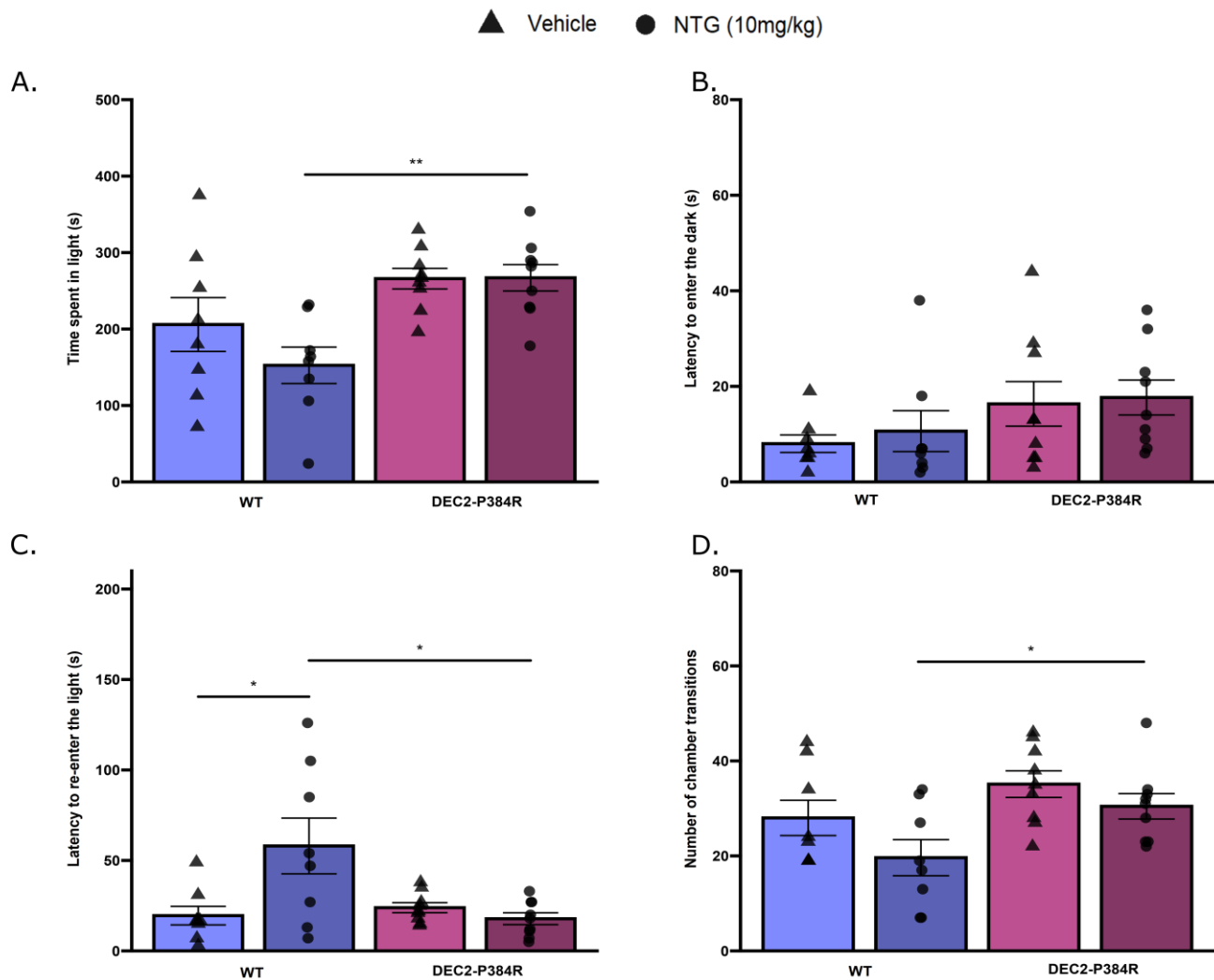
Figure 70: Altered light sensitivity in FNSS transgenic mice
A) Both WT (n = 8 per drug; $t_{9.5}$ = 0.56, p = 0.587) and hDEC2-P384R mice (n = 9 per drug; $t_{15.1}$ = 0.23; p = 0.825) given NTG (10mg/kg) do not differ in the latency to enter the dark box for the first time compared to vehicle, nor the overall time spent in the light box (**B**; $t_{12.3}$ = -1.26; p = 0.232, $t_{15.1}$ = 0.05; p = 0.960 respectively). WT mice given NTG have an increased latency to re-enter the light box after having first entered the light compared to vehicle (**C**; $t_{8.53}$; = 2.37; p = 0.043), whereas hDEC2-P384R mice do not show this distinction ($t_{15.6}$ = -1.43; p = 0.174). Mice given NTG or vehicle do not differ in the number of chamber transitions (**D**; t_{14} = -1.58; p = 0.137; t_{16} = - 1.20; p = 0.246 respectively) for both WT and hDEC2-P384R genotypes. Data represent mean ± SEM. Shapes represent data from individual animals. ** = p < 0.01, * p < 0.05 computed using Student's t-test with the Bonferroni correction for multiple comparisons applied.

7.3.9 Anxiety-like Phenotype in FNSS

As orexin has been linked to increased anxiety (Suzuki et al., 2005) and FNSS have been reported to have increased prepro-orexin expression in the hypothalamus (Hirano et al., 2018), we established whether FNSS mice display anxiety-like behaviours when compared with WT littermates using the open field test (see section 2.6.3). hDEC2-P384R and WT mice did not differ in the number of movements (398 ± 45.10 vs 409 ± 65.0 respectively, t_{22} = -0.50, p = 0.622, **Figure 71B**) around the open field arena, suggesting no overt difference in locomotor function.

hDEC2-P384R mice did not differ in the number of entries to zone 2 (19.8 ± 9.48 vs. 25.3 ± 9.92; t_{22} = -1.39, p = 0.179; **Figure 71C**), nor the distance travelled in zone 1 (1276 ± 238 inches (in) vs 1321 ± 195 in respectively; t_{22} = -0.51, p = 0.617, **Figure 71E**) . However, hDEC2-P384R mice spent significantly less time in zone 2 (28.9 ± 16.6 seconds (s) vs 47.6 ± 22.4 s; t_{22} = -2.33, p = 0.029, **Figure 71D**) than WT littermates, indicating a potential anxiety-like phenotype in hDEC2-P384R mice.
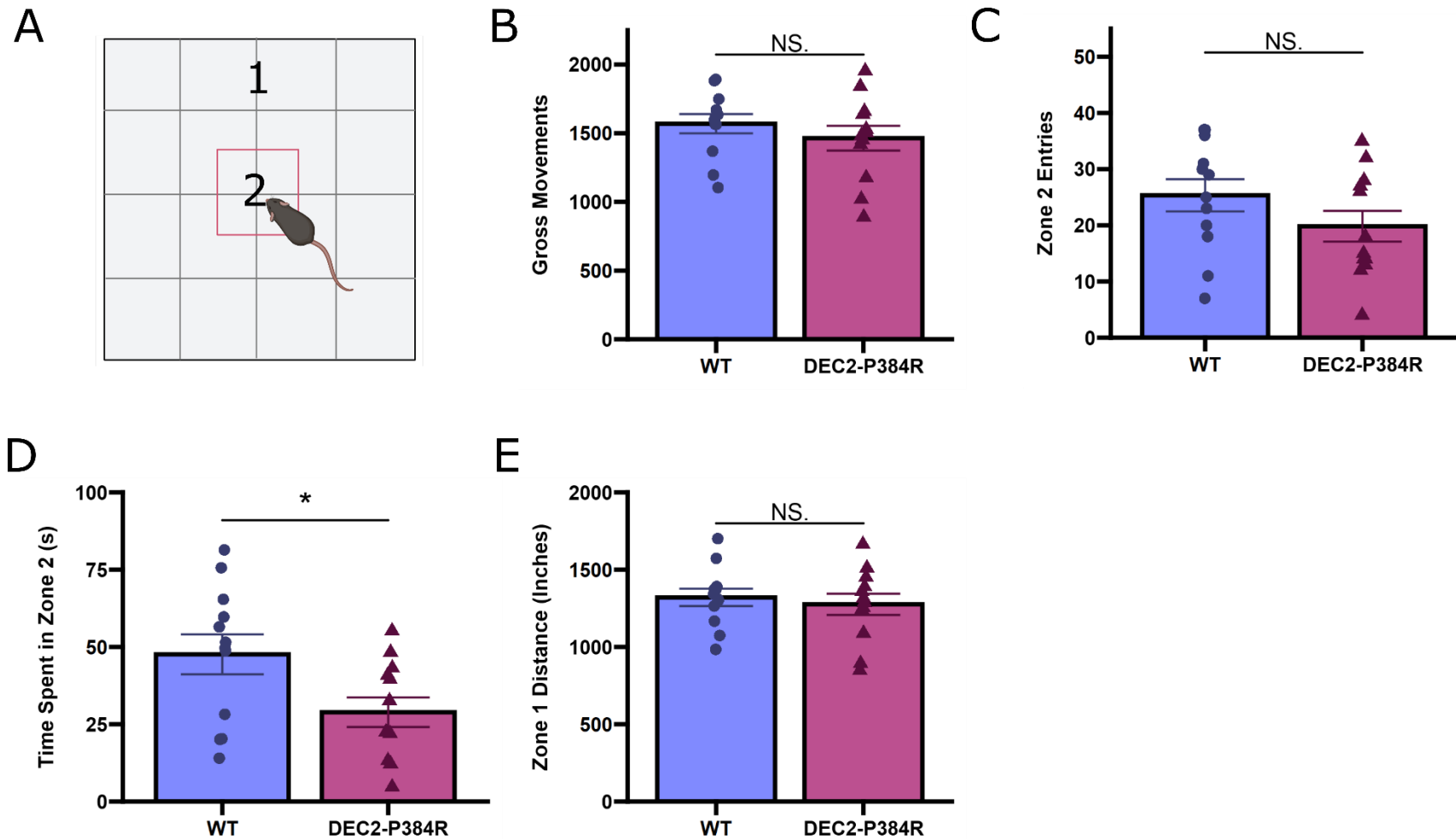
Figure 71: Anxiety-like behaviour in FNSS transgenic mice
A shows a schematic of the open field test arena highlighting the defined zones. hDEC2-384R mice (triangles; n = 12) do not differ in the number of movements around the arena (**B**; t_{22} = -0.93, p = 0.364) compared to wild-type (WT) littermates (circles; n = 12) in the open field test. hDEC2-P384R mice do not significantly differ in the number of entries into the central zone (**C**; t_{22} = -1.39, p = 0.179), or the total distance spent in zone 1 in inches (**E**; t_{22} = -0.51, p = 0.617). However, hDEC2-P384R mice spend significantly less time within the central zone (**D**; t_{22} = -2.33, p = 0.029). Data represent mean ± SEM. Shapes represent data from individual animals. NS. = not significant, * p < 0.05 computed using Student's t-test with the Bonferroni correction for multiple comparisons applied.

282

7.3.10 Altered metabolite concentrations in FNSS

Measuring changes in neural metabolite concentrations could pinpoint altered metabolic pathways in FNSS. Data were not normally distributed thus the Mann-Whitney U-test was computed, corrected for multiple comparisons, and are represented as median (IQR; see **Table 24** for descriptive and inferential statistics). NMR spectroscopy analysis revealed a significantly higher concentration of ATP ($U = 26$; $p = 0.004$), adenosine ($U = 32$; $p = 0.048$), alanine ($U = 32$; $p = 0.048$), and NAM ($U = 31$; $p = 0.031$) in FNSS brains than WT controls, whereas NAD^+ was significantly lower ($U = 4$; $p = 0.048$). After acute sleep deprivation, FNSS had higher levels of ATP than WT ($U = 50$; $p = 0.005$). There was no significant difference in other metabolites.

Table 24: Whole brain metabolite concentrations in FNSS

| Metabolite | Sleep Deprived | | | | Non-Sleep Deprived | | | |
	*h*DEC2-P384R (*n* = 6)	WT (*n* = 9)	*U*	*p*-value	*h*DEC2-P384R (*n* = 6)	WT (*n* = 12)	*U*	*p*-value
ATP	5.54 (1.14)	3.54 (2.08)	50	0.005**	8.1 (2.09)	3.82 (1.83)	26	0.004**
ADP	2.81 (2.17)	4.24 (4.20)	26	0.955	4.3 (2.44)	3.97 (4.88)	32	0.095
Adenosine	111.35 (44.98)	118.57 (20.45)	20	0.456	127.55 (39.81)	115.49 (14.57)	32	0.048*
Acetate	49.67 (3.04)	50.22 (7.60)	26	0.955	60.5 (11.81)	54.28 (11.30)	26	0.295
Lactate	909.21 (176.36)	866.94 (114.07)	34	0.456	932.92 (119.75)	125.32 (191.40)	23	0.536
Pyruvate	69.63 (11.61)	77.47 (12.06)	23	0.689	88 (14.82)	79.62 (9.10)	28	0.180
Alanine	26.29 (7.62)	27.78 (5.21)	29	0.864	39.14 (5.71)	28.1 (5.70)	32	0.048*
NAM	5.89 (1.48)	4.86 (2.95)	31	0.689	8.86 (3.54)	5.6 (2.55)	33	0.031*
NAD$^+$	2.59 (1.28)	2.56 (2.67)	20	0.456	1.89 (0.78)	2.92 (2.55)	4	0.048*
Glutamate	109.16 (17.97)	116.12 (26.27)	23	0.689	132.64 (14.63)	112.09 (14.44)	25	0.365
Creatine	104.64 (15.72)	105.4 (19.03)	29	0.864	133.15 (32.26)	112.44 (27.85)	26	0.295
Fumarate	2.49 (0.42)	2.2 (0.99)	31	0.689	3.1 (0.68)	2.67 (1.51)	21	0.730
Glutamine	108.2 (8.08)	120.14 (27.32)	23	0.689	131.69 (17.16)	125.32 (31.04)	20	0.840
PCr	42.91 (6.39)	47.07 (13.03)	26	0.955	59.29 (14.33)	50.57 (12.99)	27	0.233

Values are reported as median (IQR) and quantified as μM / mg of protein. * = $p < 0.05$, ** = $p < 0.01$ computed using Mann-Whitney *U*-test with Bonferroni correction for multiple comparisons applied.

7.3.11 Reduced Prepro-orexin Transcription Factor Levels in FNSS

To confirm previous reports of increased prepro-orexin levels in NSS mice we conducted qPCR to assess prepro-orexin mRNA transcription factor levels in hypothalamic-enriched tissue at ZT8 in hDEC2-P384R mice and WT littermates in control conditions and after acute sleep deprivation. One WT mouse was excluded due to being a significant outlier (fold expression: -1.49; final n WT-sleep-deprived = 3) as identified by Grubbs' test. Overall, there was a genotype effect on prepro-orexin mRNA levels ($F_{(1,23)}$ = 9.23, p = 0.006, η^2_G = 0.27) in that hDEC2-P384R mice had significantly less prepro-orexin mRNA relative to β-Actin compared to WT littermates (0.14 vs -0.79, t_{25} = 3.06, p = 0.005; **Figure 72**). However, sleep deprivation had no overall effect on prepro-orexin mRNA levels ($F_{(1,23)}$ = 1.12, p = 0.302, η^2_G = 0.03).
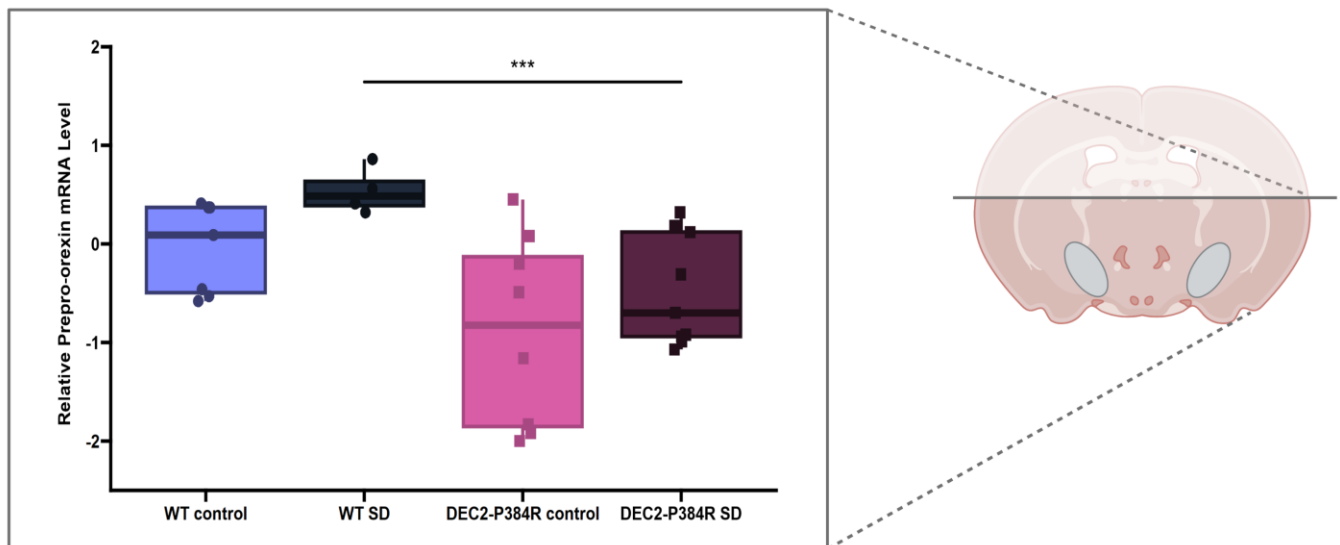
Figure 72: Decreased prepro-orexin mRNA level in FNSS at ZT8
There was no effect of acute sleep deprivation on mRNA level in hypothalamic-enriched tissue at ZT8 in hDEC2-P384R mice (squares; n = 9/8) or wild-type (WT) littermates (circles; n = 3/7; $F_{(1,24)}$ = 1.12, p = 0.302, η^2_G = 0.03). hDEC2-P384R mice had significantly less prepro-orexin mRNA level overall vs WT littermates (n = 17/11; t_{25} = 3.06, p = 0.005) as assessed by Student's t-test). Abbreviations: SD = sleep deprivation. Data are normalised relative to β-Actin and represented as log fold change and are represented as mean ± SEM. Shapes represent data from individual animals. *** = p < 0.001 computed using Student's t-test with the Bonferroni correction for multiple comparisons applied.

7.4 Discussion

Within this chapter we provided evidence for altered migraine-related sensitivity in animal models of FNSS, elucidating possible genetic influences which may curtail migraine development. Specifically, we found, under normal conditions, no alterations in cephalic and extracephalic mechanical or thermal sensitivity, or CSD susceptibility in mice harboring the P384R mutation in *h*DEC2. However, once exposed to the clinical migraine trigger NTG, FNSS mice do not develop NTG-evoked orofacial mechanical allodynia and photophobia suggestive of decreased migraine susceptibility. FNSS mice also show accompanying alterations in whole brain metabolite concentrations involved in energy metabolism and oxidation. Although, when subject to sleep deprivation, FNSS exhibit extracephalic thermal and cephalic mechanical allodynia, suggesting that they may not be immune to the nociceptive effects of sleep deprivation.

Recapitulation of the short sleep phenotype

Firstly, we confirmed that mice harbouring a mutation for FNSS exhibit the short sleep phenotype observed in human carriers. Although we used circadian locomotor activity patterns as a surrogate read out of sleep/wake cycles, rather than EEG, we observed a similar onset period and later activity offset period and an overall reduction in sleep in FNSS akin to the similar sleep onset and earlier sleep offset in human carriers (He et al., 2009). However, there are discrepancies between the current findings and previous which should be addressed.

Most notably, we identified a significant, but modestly shorter endogenous period (τ) in FNSS compared to WT. This is more akin to the shorter period seen with circadian rhythm disorders such as FASP (Xu et al., 2005). This is surprising, as although DEC2 is a clock gene, mutant DEC2 is thought to regulate sleep need by

acting as a transcriptional repressor and up-regulating orexin expression, rather than affecting circadian machinery (Hirano et al., 2018). Knockout of DEC2 results in only a subtle circadian rhythm phenotype yet considerably affects sleep architecture (Baier et al., 2014; Bode et al., 2011). Furthermore, mutations in DEC2 affect the sleep rebound after sleep deprivation in mice and humans (He et al., 2009; Pellegrino et al., 2014), suggesting that DEC2 regulates sleep homeostasis rather than the circadian clock. Given that this circadian phenotype was subtle and only apparent during DD conditions in which none of the behavioural testing was conducted, it is unlikely that this impacted on our findings and therefore the results can be attributed to changes in sleep duration.

We corroborated previous reports of overall increased activity and less sleep in *h*DEC2-P384R mice (He et al., 2009), the increase in activity was within the dark not the light phase, contrary to previous reports. This is surprising, as mice are nocturnal, and we might expect activity differences to be most apparent during the mouse's main sleep period (light phase). However, the previous EEG study reported differences in the sleep rebound only in the dark rather than the light phase. Moreover, we did not detect the longer activity period (α) reported in the original study (He et al., 2009). This could reflect differences in using PIR sensors rather than EEG to determine sleep-wake patterns. Nonetheless, the recapitulation of a short sleep phenotype herein confirms that the *h*DEC2-P384R mutation leads to FNSS, and thus is a valid translational model to probe the relationship between short sleep and migraine-related phenotypes.

Alterations in migraine-related phenotypes

This work was predicated on anecdotal evidence of increased pain thresholds in human FNSS (verbal communication from Professor Louis Ptáček, UCSF). Yet despite

a clear short sleep phenotype, the FNSS allele in mice does not appear to affect baseline orofacial or hind paw sensitivity both in the mechanical and thermal domain, nor cortical excitability, contrary to our hypothesis. This is in line with negative unpublished findings from another FNSS allele: NPSR1-Y206H (**Figure 55**).

These results are surprising yet are in line with current theories. For example, it is postulated that migraine is a sensory threshold disorder (Peng & May, 2019) in which individuals with migraine display a genetic hypersensitivity to sensory input. This theory postulates that there are phasic changes in hypersensitivity (Coleston et al., 1994; Coppola et al., 2007), and argues that differences between patients and controls in sensory thresholds may not be apparent until a trigger occurs. The findings herein support this theory, as we found differences in migraine susceptibility between FNSS and non-carriers only when exposed to the trigger NTG. In line with this, mice carrying mutations for FASP show altered sensitivity to NTG, but display no alterations in mechanical or thermal sensitivity at baseline when compared to WT littermates (Brennan et al., 2013; Strother, 2020). NTG only reliably triggers headache with migraine-like features in migraine-susceptible individuals (Afridi et al., 2004; Maniyar et al., 2014; Thomsen et al., 1994), or those with a family history of migraine (Sances et al., 2004). Thus, FNSS may be less genetically susceptible to these triggers than other individuals.

Alternatively, it could be that a differential response in FNSS is only uncovered during painful conditions like NTG or neuropathic pain states. FNSS exhibit increased orexin expression at ZT1 (Hirano et al., 2018), and as touched upon previously, prior research has demonstrated that orexin may be more beneficial in inflammatory than acute pain states (Bingham et al., 2001).

The fact that we observed no difference in CSD susceptibility was surprising. Previous work has shown an increase in CSD events in mice harbouring mutations for FASP (Brennan et al., 2013). However, we identified using power analysis that a study requires at least 10-12 per group to reliably identify differences in CSD susceptibility (see section 2.1), and yet due to technical difficulties and time constraints due to COVID-19, we conducted the study with 6-8 animals per group. Thus, we cannot rule out a difference in cortical excitability in FNSS, however based on the current data (the number of CSD events did not vary considerably across genotypes) this is unlikely. Furthermore, as there were no differences in baseline sensitivity in FNSS, and these were only revealed after a trigger, it would be interesting to conduct CSD experiments after acute sleep deprivation or NTG administration in FNSS. Previous work demonstrated an increase in CSD susceptibility following acute sleep deprivation as well as a role of metabolic pathways in this process (Kilic et al., 2018; Negro et al., 2020).

In line with our hypothesis, FNSS displayed reduced light sensitivity and higher sensory thresholds when exposed to the clinical migraine trigger NTG, as opposed to WT mice. This effect could be partly driven by the orexinergic system. FNSS have been demonstrated to have increased prepro-orexin and OX_2R mRNA levels at ZT1 (Hirano et al., 2018), and we reported in **Chapter 6** an anti-nociceptive effect of OXA on sleep-deprivation induced orofacial allodynia. Narcolepsy type 1 patients which display a depletion of orexinergic neurons and reduced CSF orexin, have an increased prevalence of migraine compared to the general population (Dahmen et al., 2003). These findings however do not align with our observation (**Chapter 6**) that acute OXA administration is unable to block the effects of acute NTG. However, we investigated acute OXA administration rather than chronic upregulation of orexin expression (OXA

and OXB), observed in FNSS. It is important to note that the effects of chronically increased orexin expression might be more complex than acute delivery of orexin peptides, and it remains unclear whether orexin may be involved in FNSS' reduced susceptibility to NTG.

Unfortunately, however, we were not able to confirm the increased orexin in FNSS as we did not observe a significant increase in prepro-orexin levels at ZT8, and in fact, we found that mice with the hDEC2-P384R mutation had lower prepro-orexin mRNA levels than WT mice. This could be explained in that we conducted qPCR on hypothalamic-enriched tissue whereas Hirano et al. (2018) extracted solely the hypothalamus. The effect of increased orexin could be largely isolated to the lateral hypothalamus as indeed this is where the majority of the orexinergic cell bodies are thought to reside (Chen et al., 1999). To assess whether this behavioural effect was indeed driven by increased orexin, future studies could pre-treat FNSS with an orexin antagonist (e.g. SB-649868; Bettica et al., 2012) before delivery of NTG. Future studies could also evaluate whether this effect might be mediated by modulation of nociceptive input to the trigeminovascular system, by assessing c-Fos immuno-reactivity in the TCC, or regions in the descending pain pathway (e.g. LC or PAG).

Moreover, it is possible that there is only increased orexin expression (not overexpression) at specific time points (e.g. ZT1) which decreases the total duration of sleep and in healthy individuals orexin levels are known to fluctuate in a circadian pattern (Taheri et al., 2000). This seems plausible given the short sleep phenotype is subtle (2-3 hours difference) in these mice, and the previous studies have reported a difference in sleep during the light phase. If prepro-orexin levels were tested at the time we observed a significant increase in activity in hDEC2-P384R mice (e.g. ZT12-16) then we might also observe such a difference.

When exposed to acute sleep deprivation mice exhibit mechanical allodynia in the periorbital region as observed in WTs, in line with the literature and findings in **Chapter 5.** Interestingly, neither transgenic line displayed mechanical allodynia in the hind paw after acute sleep deprivation corroborating previous reports (Alexandre et al., 2017; Sutton & Opp, 2014), suggesting that the effects of sleep deprivation may be head-specific, or preferentially impact trigeminal sensory processing. This is interesting as cephalic sensitivity is more directly translatable to migraine as patients more commonly report orofacial allodynia during attacks (Mathew et al., 2004; only 18% of patients report extracephalic allodynia), and the orofacial region tested corresponds to V1 dermatome of the trigeminal nerve (Bathla & Hegde, 2013), and this somatotopic organisation is conserved between mice and humans (Sawyer, 2017). This finding suggests that FNSS may not be immune to the nociceptive impact of acute sleep deprivation. This emphasizes sleep deprivation as an important factor that can influence migraine susceptibility even in those which fundamentally need less sleep.

Contrary to the notion of the specificity of the effects of sleep deprivation to the face, we observed thermal allodynia in the hind paw following acute sleep deprivation in FNSS. This is at odds to the findings in WTs in **Chapter 5 (Figure 44)** as we observed no thermal allodynia following sleep deprivation. However, previous literature has reported mixed findings for thermal sensitivity following sleep deprivation highlighting the potential variability with the hotplate assay (Alexandre et al., 2017; Yu et al., 2023). Notably, responses to the hotplate show significant circadian variation (Ramge et al., 1999), and are subject to variation when at lower temperatures (e.g. 50°C vs 55°C; Plone et al., 1996), and sensitive to factors such as posture and handling (Berge, 2011). These factors could explain the discrepancy herein. Moreover, in this experiment we did not include WT littermates as a control, therefore we are

unable to ascertain whether in this experiment thermal allodynia could also be observed in WTs. Nonetheless these findings demonstrate that FNSS may not be immune to the effects of acute sleep deprivation.

Alterations in metabolites

It is proposed that there is an imbalance between mitochondrial function and increased energetic demand in people with migraine which may contribute towards migraine susceptibility (Grech et al., 2021). Herein, we demonstrated that FNSS have increased whole brain ATP, the so-called "energy currency" of cells, both at baseline and after acute sleep deprivation, suggesting increased free energy supply. This could render them less susceptible to migraine triggers in line with the behavioural observation of lack of NTG-evoked sensitisation observed herein. This is not surprising, as the circadian clock and alterations to sleep have been shown to affect metabolism (Panda, 2016). Although the tissue for the metabolic analysis was taken at the same time as the prepro-orexin mRNA analysis, and we observed no increase in prepro-orexin levels, it is still possible that these effects are due to increased orexin previously observed in FNSS (see section above for a commentary on why we might not have observed increased prepro-orexin herein). Indeed, orexin has been shown to be involved in mediating energy homeostasis (Goforth & Myers, 2017). Future studies could investigate ATP levels in the hypothalamus specifically, as ATP levels were shown to rise after sleep deprivation in the hypothalamus but not elsewhere in the brain (Dworak et al., 2011). However, FNSS were not able to buffer the nociceptive effects of acute sleep deprivation, suggesting that mechanisms other than energy metabolism may be underlying this effect.

ATP levels show a surge in the initial hours of sleep (Dworak et al., 2010). This surge is dependent on sleep but not time of day since acute sleep deprivation prevents

this increase in ATP. Dworak et al. (2010) observed a positive correlation between ATP and NREM delta power during sleep. FNSS have increased delta power during NREM sleep (He et al., 2009), thus alternatively, the increase in ATP may simply be an artefact of the increased NREM depth in FNSS.

We also observed that adenosine levels were higher in non-sleep-deprived *h*DEC2-P384R mice than WT. This aligns with the proposal of FNSS being able to cope with higher homeostatic sleep pressure, with adenosine postulated to be the neurophysiological correlate of sleep pressure (Peng et al., 2020). Increased adenosine may reflect increased degradation of ATP, and a decrease in the availability of cellular energy store (Bellesi et al., 2018) – although our ATP findings would refute this claim. Higher serum adenosine levels have been observed during migraine attacks (Guieu et al., 1994, 1998) and in preclinical models, adenosine has been shown to have antinociceptive properties (Goadsby, Hoskin, et al., 2002; Gong et al., 2010; Taiwo & Levine, 1990; Yoon et al., 2005). These findings could reflect a compensatory mechanism and anti-nociceptive function of adenosine.

We revealed alterations in other metabolites including an increase in NAM and alanine, and a decrease in NAD^+. NAM is thought to be protective against oxidative stress by restoring glycolytic rates via increasing ATP levels (de Picciotto et al., 2016; Rovito & Oblong, 2013; Villeda-González et al., 2020). Indeed, direct administration of NAM reduces oxidative stress in animal models (Turunc et al., 2013). The lower NAD+ levels observed herein may reflect that NAD is a signalling molecule which is consumed in reactions and donates NAM as a by-product (Poljsak & Milisav, 2016).

Alanine and related enzymes are also thought to be markers of anti-oxidative stress (Yamada et al., 2006). Migraine triggers including sleep deprivation are capable of generating oxidative stress (Borkum, 2016), and pain may be exacerbated by

oxidative stress (Shim et al., 2019). Thus, these results could also reflect a compensatory or protective mechanism of anti-oxidative pathways in FNSS. When energy demand is high (such as under sleep deprivation conditions), nitrogen is transaminated to pyruvate to form alanine by the enzyme alanine aminotransferase (ALT), thus greater ALT would result in greater alanine (Diab & Limami, 2016), which may also explain why we found increased alanine. Indeed, ALT is increased following sleep deprivation in rats (Everson et al., 2005; Hou et al., 2023).

7.4.2 Limitations & future directions

One consideration is that whilst FNSS mice were exposed to a standard six-hour sleep deprivation protocol after optimisation in WT mice, the altered sleep architecture in FNSS may result in a differential response and may need further characterization and optimization in FNSS. Due to COVID-19 we were unable to record EEG in these mice to confirm this. However, given we observed the short sleep phenotype in the dark phase and not during the light, this would suggest that hDEC2-P384R mice are sleeping during the light phase to the same extent as WTs. Sleep deprivation was conducted during the light phase when both transgenic lines did not differ in sleep amount. Moreover, as mentioned previously, pilot work has identified that EEG recording, and passive infrared sensors are comparable when measuring general activity patterns (**Figure 60**).

Another interesting question is the age-dependency of these effects. Dong et al. (2022) discovered age-related changes in Aβ accumulation in hDEC2-P384R transgenic mice, with an initial increase at three months followed by a significant decrease at six months compared to WTs. It is plausible that over time there is increased protection against migraine-related phenotypes in FNSS, as NREM sleep shows age-related decline in healthy individuals (Dijk et al., 2010). Generating Cre-

dependent FNSS mouse models that can be modulated conditionally and temporally will be necessary to address this possibility.

A potential avenue for future research is investigation of glycogen metabolism in FNSS, as glycogen has been linked to both headache and sleep disturbances (Petit et al., 2021). Glycogen is degraded to generate ATP during increased energy demand (Kong et al., 2002). Depletion of astrocytic glycogen stores during sleep deprivation causes transient decreases in cellular energy charge, resulting in increased adenosine (Bellesi et al., 2018). Adenosine release modulates slow wave activity as a function of sleep need in response to decreases in metabolic supply (glycogen stores) and promotes sleep onset (Peng et al., 2020). Sleep is thought to replenish these brain glycogen stores during NREM sleep (Scharf et al., 2008). Unfortunately, due to the largely overlapping spectra of glycogen making it difficult to identify individual peaks in glycogen concentration we could not reliably analyse glycogen with the NMR method used herein. That being said, key metabolites along the glycogen pathway (adenosine, ATP) were explored (Greenberg et al., 2006) and revealed key alterations. Future studies could investigate glycogen in FNSS using methods suitable for quantification of glycogen (e.g. gas chromatography mass spectrometry - GC/MS).

Additionally, we only explored one of several existing genetic models of FNSS (see **Table 22**). Exploring these lines or crossing different lines to create more extreme phenotypes may shed light on further underlying genetic mechanisms related to sleep and migraine.

Migraine is co-morbid with anxiety and depression symptoms (Peres et al., 2017) and patients suffer with cognitive-related symptoms such as confusion and memory problems (Gil-Gouveia & Martins, 2019). We observed potential anxiety-like behaviour in FNSS as *h*DEC2-P384R mice spent significantly less time in the central

zone of the open field arena. Only one study has investigated emotion-related symptoms in NSS, and observed increased subclinical hypomania compared to those with conventional sleep length (Monk et al., 2001). Although this could reflect that the short sleepers in this study could have been facultative short sleepers and were in fact sleep deprived as a result of their short sleep duration, leading to increased elevated mood (Wright, 1993). Given these findings, it would be interesting to assess cognitive and emotional functioning in human FNSS both at baseline and in response to sleep deprivation. This could provide important insight into the associated cognitive symptoms of migraine as well as have wider implications for emotional disturbance following sleep deprivation.

Individuals reporting FNSS are rare. This is likely since these individuals do not commonly report ill-effects from their short sleep duration and are therefore underrepresented in the literature. In fact, FASP family reporting mutations in CK1D were only identified after observation of comorbid familial migraine (Brennan et al., 2013). Instead of studying such rare mutations, human studies investigating the shared genetic influence between migraine and sleep, for example genome-wide association studies (GWAS), could provide important insight into this relationship. A GWAS study revealed that liability to experience insomnia symptoms increased the risk of migraine (Daghlas et al., 2020). Future GWAS studies could investigate the genetic link between sleep duration propensity and migraine risk.

7.4.3 Clinical Implications

These findings indicate that there is merit in improving sleep to protect against migraine. FNSS display increased NREM depth, cope with increased homeostatic sleep pressure, and exhibit reduced susceptibility to migraine triggers. Therefore, there is the potential for therapeutic impact solely by focusing on improving sleep via

pharmacological or behavioural interventions (see section 8.2 for a detailed discussion on this).

7.4.4 Conclusions

These findings have provided important mechanistic insight into migraine susceptibility, supporting genetic interactions between FNSS alleles and migraine. Although this is a rare genetic mutation, these data highlight a link between sleep regulation, metabolic pathways, and migraine pathophysiology.

Chapter 8: General Discussion

It has long been established that there is a link between sleep and migraine (Dodick et al., 2003; Holland, 2014; Liveing, 1873; Romberg, 1853), however this relationship remains mainly speculative, and establishing a causal or mechanistic relationship remains to be determined. In this thesis, we hypothesised that dysregulation of shared neural networks regulating sleep-wake and nociception results in enhanced pain perception and contributes to migraine attack initiation and susceptibility. More specifically, we predicted that key brainstem and diencephalic structures regulate both sleep-wake cycles and migraine biology altering thresholds for attack initiation. Utilising data from human patients and *in vivo* experiments we were able to support this overarching hypothesis. A summary of the key findings is discussed below.

8.1 Key Findings

Firstly, we established in a meta-analysis that people with migraine report poorer subjective sleep quality as measured by the PSQI than healthy individuals, an effect larger in those with chronic migraines. As measured by PSG, adult patients exhibited significantly less REM sleep compared to healthy controls, whereas pediatric patients had significantly reduced total sleep time, shorter sleep onset latency, and more wake than healthy controls as evidenced by small to large effect sizes. This suggests that migraine patients may face significant disturbances in subjective and objective sleep and highlights the potential importance of assessing and treating sleep disturbances in migraine clinically. Indeed, going to sleep is a commonly reported coping strategy for migraine attacks (Kelman & Rains, 2005) and whilst not directly tested in this thesis, treating sleep disruption in patients may potentially have some benefit in migraine. Although this remains to be elucidated.

Developing this, we explored the complex relationship between sleep and migraine further using Bayesian modelling and found that fragmented sleep, or deviation from typical sleep duration, was associated with a greater likelihood of a migraine attack occurring the next day, whereas overall sleep duration was not. Conversely, simply experiencing a migraine attack did not predict altered sleep duration. Instead, having an intensely painful attack predicted increased sleep duration. This suggests that the poor sleep experienced by migraine patients could be a potential risk factor for migraine attacks, in line with previous literature on sleep as a self-reported trigger for attacks (Kelman, 2007), and other findings demonstrating fragmented sleep as a predictor of next-day pain and migraine (Bertisch et al., 2019; Iacovides et al., 2017; Smith et al., 2007). This has also shed light on this relationship in that the poor sleep we identified in the meta-analysis is unlikely to be caused by the attacks themselves, as attacks did not predict alterations in subsequent sleep duration.

Utilising mouse models, we demonstrated that sleep deprivation leads to orofacial allodynia, a commonly reported symptom in migraine patients (Bigal et al., 2008) and neuronal activation in the PAG – a descending modulatory pain structure (Eippert & Tracey, 2014) which is implicated in migraine pathophysiology (Knight & Goadsby, 2001; Raskin et al., 1987). We further showed that sleep deprivation-evoked allodynia can be recovered with administration of a wake-promoting neuropeptide synthesized in the LH: OXA. Conversely, another arousal-promoting substance – caffeine was unable to recover this behavioural phenotype, suggesting that the antinociceptive effects could be partly independent from those mediating arousal. This finding supports a role of the hypothalamic orexinergic system in migraine (Holland & Goadsby, 2007) building on a growing body of data that OXA may be a potential novel therapeutic target (Bartsch et al., 2004; Holland et al., 2005).

Lastly, we explored potential genetic mechanisms underlying pain and migraine susceptibility in FNSS mice harbouring the P384R mutation in *h*DEC2. We firstly confirmed the short sleep phenotype in FNSS in-house before exploring various migraine-related readouts. FNSS transgenic mice demonstrated increased resistance to the clinical migraine trigger NTG, and increased concentrations of metabolites involved in energy metabolism in baseline conditions. Whilst we do not have direct evidence that these metabolites are involved in the potential reduced susceptibility to migraine triggers, the findings are suggestive of a protective role of familial short sleep duration and altered energy metabolism in migraine pathophysiology. This is in line with research highlighting perturbed metabolism in headache pathophysiology (Grech et al., 2021; Kilic et al., 2018; Santos et al., 2012; Uncini et al., 1995), as well as the involvement of metabolites such as alanine and NAM in buffering oxidative stress (de Picciotto et al., 2016; Rovito & Oblong, 2013; Villeda-González et al., 2020). Interestingly, circadian disruption can result in migraine-related phenotypes as observed in FASP mice with mutations in the CLOCK gene CK1δ (Brennan et al., 2013; Strother, 2020), and loss of Cry1 and Cry2 (repressive component of the TTFLs) results in increased pain responses (Wei et al., 2022). The findings in this thesis extend this by additionally demonstrating that genes which regulate homeostatic sleep need (DEC2; Hirano et al., 2018) may also impact migraine-related processing.

Taken together, the findings in this thesis highlight sleep disruption as a potential risk factor for migraine likelihood and re-emphasize the importance of sleep for migraine and health more generally. Whilst furthering our understanding of migraine, these findings also have the potential to be translated into novel pharmacological and behavioural interventions for migraine and other headache

disorders, as well as pain more generally. These will be discussed in the following section.

8.2 Therapeutic Implications for Migraine

8.2.1 Pharmacological Interventions

Orexin-A

In **Chapter 6** we identified a novel pharmacological target for sleep-deprivation-induced migraine-related allodynia as OXA was able to recover mechanical allodynia induced by sleep deprivation but not by the clinical trigger NTG. This suggests that sleep deprivation may induce alterations in sleep-wake regulating neural systems. Targeting this system may provide therapeutic benefit for migraine and other headache disorders in agreement with previous preclinical literature demonstrating an antinociceptive effect of OXA and OX_1R activation (Bartsch et al., 2004; Holland et al., 2005; Yamamoto et al., 2002; Yamamoto, Saito, Shono, Aoe, et al., 2003). A previous study explored the efficacy of a DORA for migraine with a negative outcome (Chabi et al., 2015), yet the findings herein suggest that increasing OXA specifically may have therapeutic benefit.

These findings suggest that cutaneous allodynia evoked by the trigger NTG and that induced by sleep deprivation may be governed by different underlying mechanisms and/or intracellular signalling cascades. This might be explained in that in patients triptans tend to be more effective treatments when symptoms are mild e.g. before the development of central sensitisation (Burstein et al., 2004). Thus, treatments may be differentially effective in response to various triggers. In this case it is possible that sleep deprivation is a less potent trigger than NTG. In human patients, NTG triggers attacks in 80% of individuals (Thomsen et al., 1994), whereas

302

only ~50% of patients report sleep disturbance as a trigger (Kelman, 2007). This has potential implications for the use of OXA as a treatment, as like triptans – it may prove more efficacious when symptoms are mild.

Another consideration is that OXA is arousal-promoting thus chronic administration could lead to unwanted side effects such as insomnia, particularly if delivered at night. This is important as we have shown here that disruption to sleep could trigger attacks, and further disruption could lead to chronification or exacerbation of migraine. Chronopharmacology studies need to be conducted in order to establish the optimum delivery time for OXA or selective OX_1R agonists for migraine treatment (Salamatova & Packard, 2021). This is important as the efficacy of other migraine treatments such as onabotulinumtoxin A have recently been shown to potentially vary in line with circadian phase (Packard et al., 2021), with treatment most effective in the afternoon. Although, this study did not factor in individual chronotypes which may also affect treatment efficacy (Viticchi et al., 2019), as chronotype has been shown to predict clinical response to treatment in other conditions (Dallaspezia et al., 2018; McCarthy et al., 2019). Future research may be required to provide a comprehensive understanding of OXA delivery according to circadian phase and chronotype.

Novel drug delivery systems have been developed to address this issue. For example, controlled-onset extended-release (COER) verapamil – a calcium channel blocker used in the treatment of cluster headache was specifically designed for bedtime dosing and morning release (Ranganathan et al., 2021). Given that most migraine attacks occur in the early hours of the morning upon awakening (Alstadhaug et al., 2008), COER systems could be used to deliver OXA-related therapies, ensuring less impact of OXA on sleep. COER drugs are already widely used to treat insomnia (e.g.

zolpidem; Moen & Plosker, 2006; Moline et al., 2021), suggesting the practical utility of this system. Moreover, the findings in this thesis suggest the utility of OXA as an acute treatment. However, whether OXA would be most beneficial as a preventative is unclear.

Lastly, OXA was delivered i.p. in this thesis. Whilst this can cross the BBB to some extent (Kastin & Akerstrom, 1999), OXA may have poor BBB penetrance particularly in small doses. Larger doses could lead to unwanted side effects. Intranasal on the other hand bypasses the BBB and importantly has rapid access to the TG and trigeminal nerve (Dhuria et al., 2010), with direct penetration to the CNS including the hypothalamus, meaning that lower concentrations would be required and thus potentially less side-effects. Intranasal OXA has been shown to offset the effects of sleep deprivation on cognition in primates (Deadwyler et al., 2007). Whilst the pharmacology of OXA administration may be different from mouse to human, clinically, intranasal OXA has been successful with respect to treating narcolepsy with cataplexy (Weinhold et al., 2014), suggesting that this could be re-purposed as a treatment for migraine. Moreover, as highlighted previously, most migraine attacks occur in the early hours of the morning (Alstadhaug et al., 2008) thus delivering treatment at this time when arousal is high (Silver & LeSauter, 2008), and attacks have already begun, is unlikely to have significant benefit. Delivering fast-acting intranasal OXA could prove beneficial in this case.

Sleep-specific targets

We demonstrated herein that patients with migraine display problems with sleep and that this is potentially involved in increasing the likelihood of migraine attacks. Therefore, pharmacological treatments which aim to improve sleep specifically may be beneficial in addressing any sleep disturbances. For example, those used for

treating insomnia (e.g eszopiclone, Lemborexant) may prove fruitful (De Crescenzo et al., 2022). Of particular relevance to the findings in this thesis, REV-ERBα receptor agonists have been shown to be effective at regulating both sleep and metabolism in mice (Amador et al., 2016; Ripperger & Albrecht, 2012; Solt et al., 2012; Wang et al., 2020); however, to our knowledge they have not at present been tested for sleep disorders and/or migraine in humans.

Whilst we did not directly explore the impact of melatonin on migraine in this thesis, in terms of improving sleep problems and reducing the likelihood of sleep disturbance precipitating attacks, the findings highlight the potential of melatonin as a preventive treatment for migraine. Melatonin has already shown promise in treating migraine (Eli & Fasciano, 2006; Gagnier, 2001; Long et al., 2019), as well as in subjective sleep quality in primary insomnia (Lemoine et al., 2011). Although exogenous melatonin does not appear to have a tangible effect on NREM or REM sleep architecture, it has been shown to increase sleep efficiency (Brzezinski et al., 2005), TST, and decrease SOL (Ferracioli-Oda et al., 2013). Herein, we noted decreased TST in children with migraine (see **Chapter 3**), thus melatonin could help to increase sleep duration and sleep efficiency, which may have concomitant benefits for migraine. Indeed, these results raise the question as to whether the observed benefits of melatonin are due simply to improvements in sleep. Although, in children, melatonin and a subsequent nap were independently associated with greater headache benefit (Gelfand et al., 2020), suggesting melatonin treatment and behavioural sleep interventions may be effective when combined. Although, recent meta-analytic evidence contests the efficacy of melatonin for insomnia (De Crescenzo et al., 2022), therefore further research should explore the efficacy and mechanisms of melatonin for treating sleep disorders.

<u>Metabolic targets</u>

In this thesis we demonstrated that FNSS carrying the *h*P384R mutation in DEC2 were less responsive to the migraine trigger NTG. These mice also had increased whole brain ATP both at baseline and following acute sleep deprivation In line with reports on perturbed energy metabolism and demand in headache (Grech et al., 2021), increased ATP levels in FNSS could be a potential protective mechanism leaving more energy to buffer metabolic demand after sleep deprivation. Although at present this is speculative and blocking ATP in FNSS will be required to determine specificity of this effect. ATP is commonly present in many foods including fish and meat, and supplementing ATP is therefore a potential safe target for migraine (Herda et al., 2008). ATP supplements are commonly used by athletes to maintain ATP levels during high intensity exercise (dos Santos Nunes de Moura et al., 2021). Although, these supplements may have limited bioavailability (Arts et al., 2012), and future research is required to determine their effects (if any) on brain metabolism.

8.3 Behavioural Interventions

Whilst pharmacological interventions for sleep may be useful for some patients, they are often accompanied by a myriad of side effects as well as issues of tolerability (Morin & Kwentus, 1988). Behavioural sleep interventions – and 'sleep hygiene' guidance on the other hand exhibit few medical contraindications, are typically cost-effective, and are relatively easy to administer where appropriate. Sleep hygiene advice may include limiting daytime naps, eliminating activities in bed (watching television, reading), scheduling a consistent bedtime, and limiting food intake to >4 hours before bedtime. Moreover, digital cognitive behavioural therapy (d-CBT) delivered through smartphones or personal computers has shown promise in treating insomnia (Espie et al., 2019; van Straten et al., 2018). However, it is important to note that these

interventions may be more difficult to implement in patients with chronic migraine especially as they may be more likely to experience mental health co-morbidities (Buse, Silberstein, et al., 2013), and this could mean adherence to sleep interventions is low or contraindicated.

Whilst only a small number of studies investigating behavioural sleep interventions for migraine have been conducted (Sullivan et al., 2019), these have shown promise in improving sleep and migraine frequency (Bruni et al., 1999) in adults and children. As well as reducing headache frequency, intensity, and related disability (Sandoe et al., 2019) they may additionally help to revert chronic conditions to episodic (Calhoun & Ford, 2007) lessening the burden for migraine patients.

Given that total sleep time was shown not to be a strong predictor in migraine attacks in this thesis, interventions may focus mainly on ensuring sleep continuity. This could be achieved by limiting food and drink as well as environmental noise, and external conditions such as temperature and light, as well as limiting caffeine and alcohol consumption (Muzet, 2007; Shimura et al., 2020). This is particularly important for migraine in which light and other sensory stimuli may be abnormally processed (Goadsby, Holland, et al., 2017b; Harriott & Schwedt, 2014).

Moreover, the findings in this thesis highlight that caffeine may be involved in exacerbation of migraine attacks (see **Chapter 6**). Not only does oral administration of caffeine affect sleep quality and objective sleep measures (Drake et al., 2013; O'Callaghan et al., 2018; Panagiotou et al., 2019) which in itself could precipitate migraine attacks, but we demonstrated that it may lead to orofacial allodynia. Although the clinical picture of caffeine in migraine is complex as it is often used as an analgesic to treat headache (Lipton et al., 2017), is implicated as a precipitating factor (Scher et al., 2004), and may be related to withdrawal (Alstadhaug et al., 2020).

Moreover, it is important to note the impact of one large intraperitoneal dose in mice never exposed to caffeine previously may be vastly different to that a human with regular exposure to oral caffeine, thus further studies with chronic delivery and oral administration should be conducted. However, the findings herein suggest that abstaining from caffeine may be important in migraine prevention.

Moreover, given that FNSS have increased NREM depth and reduced susceptibility to the migraine trigger NTG, it is possible that interventions targeting NREM depth and continuity could prove fruitful in migraine. One such method is closed-loop auditory stimulation in which small bursts of pink noise are played timed to the up-states of the slow-oscillation by using slow oscillation tracking algorithms such as a phase-locked loop (Ngo et al., 2013; Santostasi et al., 2016). Whilst this has been shown to not have a direct impact on sleep architecture in terms of NREM duration (Stanyer et al., 2022), it is possible that it could have an impact on NREM depth as measured by delta power increases. This is a relatively cost-effective, non-invasive safe therapy, and numerous portable commercial devices are available to the wider public (e.g. Dreem; Debellemaniere et al., 2018) suggesting the utility of this as a treatment for sleep in patients with migraine.

Alternatively, although we do not know whether the reduction in REM observed herein (**Chapter 3**) is a consequence of migraine or abnormal migraine-related CNS activity which precedes the pain phase, it is possible that specifically boosting REM sleep could be efficacious in migraine. Systems have been developed to boost specifically REM sleep via theta activity (3-7 Hz) may prove fruitful (Harrington et al., 2021). Furthermore, there is the potential to boost spindle activity during NREM sleep (Ngo et al., 2019), which may impact impaired thalamo-cortical connectivity and cortical excitability observed in migraine (Gollion, 2021).

These results also suggest that shift work or jet-lag may be particularly damaging for those susceptible to migraine as we identified a deviation in sleep duration as a predictor of migraine. This is in agreement with studies highlighting the potential impact of shift-work on migraine (Sandoe et al., 2019), although there is a lack of literature in this area (Leso et al., 2020). As well as studies demonstrating the adverse effects of jet lag paradigms in mice on migraine-related phenotypes (Strother et al., 2020). Thus, these results highlight the importance of sleep schedule consistency as well as circadian hygiene, as this has also been shown to predict adverse health outcomes (Chaput et al., 2020).

One way in which sleep schedules may be maintained is by controlling exposure to artificial light. Migraine patients tend to exhibit more extreme chronotypes (Li et al., 2023; van Oosterhout et al., 2018) and may therefore be exposed to light incongruent with their circadian cycle. Whilst currently contested, blue light in the evening (mostly from smartphones and other devices) has been shown to have a considerable impact on subjective sleep quality (Wahl et al., 2019) and the proportion of SWS (Ishizawa et al., 2021). Of relevance, for migraine, extreme morning types may be more affected by blue light exposure (Siemiginowska & Iskra-Golec, 2020). Recent evidence suggests that the sleep-altering effects of blue light could be ameliorated with the use of blue-light-suppressing glasses (Guarana et al., 2021) or by simply increasing exposure to morning light (Zerbini et al., 2020). Small to medium effect sizes have been reported for TST and sleep efficiency with blue-light-blocking glasses whereas large effect sizes have been reported for subjective sleep quality (Shechter et al., 2020). Other trials have reported no effect on objective sleep measures (Bigalke et al., 2021). Interestingly, exposure to blue light at night has been shown to suppress energy

metabolism the following morning (Kayaba et al., 2014), highlighting the potential interplay of metabolism, sleep, and migraine (Wahl et al., 2019).

Whilst adjusting daily light exposure may be beneficial, ensuring that light exposure is tailored throughout the year might also prove useful in migraine sufferers. Recent evidence on the seasonality of sleep architecture demonstrated an increase in REM sleep and shorter REM sleep latency during the winter months in Europe where light levels are low (Seidler et al., 2023). This is interesting as January has been reported as being the most likely month for higher frequencies of migraine attacks in a European sample (Cugini et al., 1990). Thus, by increasing exposure to light in the winter either by spending more time outdoors or artificial light may prove effective in reducing migraine attack occurrence.

8.4 Limitations

8.4.1 Differences Between Murine and Human Sleep

The work in this thesis has provided novel understanding of the relationship and mechanisms of sleep and migraine. However, in investigating this relationship we relied partly on preclinical mouse models. Whereas circadian regulation is largely conserved across mice and humans (Foster et al., 2020; Takahashi, 2017) , sleep shows key differences and therefore there are limitations when considering the findings in this thesis.

Most notably – mice are nocturnal and exhibit a τ shorter than 24 hours (Finger & Kramer, 2021) and sleep is polyphasic and highly fragmented in mice compared to the quasi-monophasic sleep of modern humans (Stampi, 1992; see section 8.4.1). That being said, evidence exists that human populations isolated from modern civilisation exhibit highly polyphasic sleep (Petre-Quadens, 1983), and humans housed under a

shorter photoperiod may revert to a biphasic sleep pattern (Wehr, 1992), suggesting that underlying sleep patterns may be more similar to that of a mouse. However, in wild mice there may be substantial variations in the sleep patterns compared to laboratory mice. For example, wild mice are not explicitly nocturnal as they exhibit feeding behaviour that is predominantly, and sometimes exclusively diurnal (Daan et al., 2011). In fact, feeding behaviour is likely regulating sleep/wake cycles as restricting feeding times in laboratory mice can impact on the coupling of central and peripheral circadian clocks (Damiola et al., 2000).
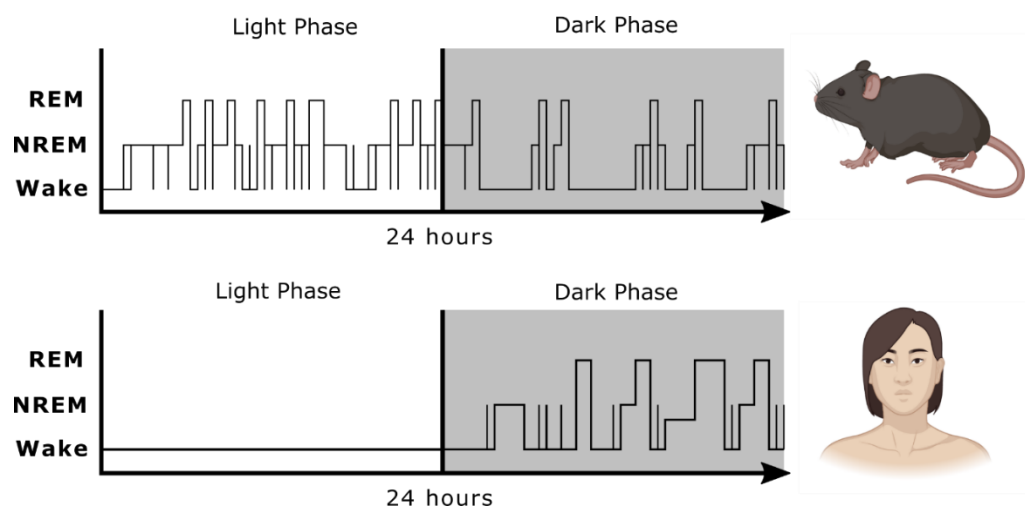
Figure 73: Murine versus human sleep
A comparison of human versus laboratory mouse sleep during light and darkness over a 24-hour period. Mice typically show polyphasic, fragmented sleep, which is distributed across light and dark phases, whereas humans exhibit monophasic sleep, with a continuous period of sleep cycles in the dark phase. Abbreviations = REM = rapid-eye-movement sleep, NREM = non-rapid-eye-movement sleep.

What's more, melatonin is released primarily during the dark in humans and rodents, but is absent in the majority of common laboratory mouse strains e.g. (C57BL/6J; Kennaway, 2019). However, mice which are melatonin-proficient have been compared to melatonin deficient strains and there are no noticeable differences in circadian or sleep patterns (Stehle et al., 2002), suggesting that this is unlikely to have an impact on translation of findings from mice to humans. Although, melatonin does show promise as an anti-migraine therapy (Eli & Fasciano, 2006; Long et al.,

2019) and nocturnal melatonin levels are decreased in patients with migraine (Brun et al., 1995), suggesting a link between melatonin and headache, thus we have to consider that the current findings could be different in melatonin-proficient mice.

Additionally, night-time light exposure provokes awakening in humans, whereas it triggers sleep and suppresses activity in nocturnal rodents – a phenomenon known as negative masking. Thus, the use of nocturnal rodents for translational studies must be considered with respect to these limitations (Mendoza, 2021). Moreover, the intrinsic features of circadian oscillators including the expression of key clock genes may differ between nocturnal and diurnal species (Tournier et al., 2007). For this reason, studying the relationship between sleep and migraine in a diurnal rodent such as Nile rats (*Arvicanthis niloticus)* in the future may provide further insight into underlying mechanisms (Refinetti, 2004).

Despite these differences, there are many commonalities between mouse and human sleep and there are strengths to using preclinical models of sleep. Firstly, they allow controlled investigation of light. Modern humans are exposed to artificial light which is out of sync with the natural circadian rhythm resulting in circadian rhythm disorders and sleep disorders (Tancredi et al., 2022). By studying mouse models, we can study the underlying circadian machinery and sleep architecture by closely controlling and manipulating light levels. Moreover, genetic sleep models are not possible in humans, and studying rare mutations such as *h*DEC2-P384R which results in FNSS can provide important insight into underlying neurobiological mechanisms.

8.5 Implications for Other Headache Disorders

The current research findings primarily focused on their relevance to migraine, but their potential applicability extends to other primary headache disorders as well. For instance, cluster headaches exhibit a distinct circadian and circannual pattern,

with most attacks occurring at the same time each day and bouts recurring during specific times of the year, such as spring or autumn (Benkli et al., 2023; Pergolizzi et al., 2020). Although this remains a topic of debate, individuals with cluster headaches may also experience a reduction in REM sleep, mirroring the findings observed herein in migraine patients (Barloese et al., 2015, 2015). Furthermore, tension-type headache patients also report sleep abnormalities and identify sleep as a potential trigger for their headaches (Cho et al., 2019). In addition, both cluster headache (Wilbrink et al., 2017) and chronic tension-type patients (de Tommaso et al., 2014) exhibit cutaneous allodynia which has been associated with duration and severity of headaches (B.-S. Kim et al., 2019). Notably, they share many of the same risk factors for attacks as migraine patients, hinting at a potential common underlying pathophysiology.

Considering these connections, our findings on sleep fragmentation, and acute sleep deprivation and cutaneous allodynia therefore may also be applicable to other headache disorders. Future studies should explore the relationship between sleep and other primary headache disorders as well as investigate the potential efficacy of sleep interventions in these populations.

8.6 Future Research Directions

Whilst the current study has unravelled potential mechanisms underlying the association between sleep and migraine, there is work remaining to further understand this relationship and neural basis.

We demonstrated in **Chapters 5 and 6** that sleep deprivation in mice leads to migraine-related phenotypes in mice. Whilst we have discussed the translational value of the current studies (see section 8.4.1) directly assessing the impact of sleep deprivation on migraine symptoms in humans could be valuable. This could be

achieved with selective sleep disruption paradigms, such as slow wave or REM sleep suppression using acoustic stimuli (Groeger et al., 2014).

Moreover, as touched upon in **Chapter 3**, one interesting aspect of sleep microstructure which has never been investigated and may be altered in migraine is sleep spindles. Their relevance for migraine is that sleep spindles are thought to serve as a gating mechanism for nociceptive information during sleep and integration of multisensory inputs more generally (Fernandez & Lüthi, 2020). Indeed, recent evidence suggests they may in fact facilitate transmission of nociceptive information to the cortex (Claude, Chouchou, Prados, Castro, Blay, et al., 2015), suggesting a plausible mechanism for the observed effect of excess sleep on migraine attacks (Inamorato et al., 1993). Furthermore, alterations in sleep spindles are observed in many conditions including epilepsy (Yu et al., 2018) and schizophrenia (Wamsley et al., 2012) in which cortical inhibition-excitation is altered, and neuronal hyperexcitability underpins a key theory of migraine (Aurora & Wilkinson, 2007). Given the array of multisensory symptoms during migraine (photophobia, phonophobia, and sensitivity to touch), it is plausible that thalamic sleep spindles are altered or dysfunctional in migraine. However, this has not yet been investigated in a migraine sample.

Moreover, based on the preclinical data (**Chapters 5-7**) and clinical findings in **Chapter 4**, the data in this thesis justifies a clinical trial which investigates the efficacy of sleep interventions on migraine frequency and/or intensity.

8.7 Conclusion

Migraine is a highly disabling condition affecting over one billion people. Poor sleep is equally prevalent with humanity thought to be in the midst of a global sleep epidemic. The findings of this thesis have shed light on the relationship between sleep

and migraine, highlighting poor sleep as a potential risk factor for migraine likelihood, and identifying pharmacological targets and possible genetic mechanisms underlying sleep regulation which may curtail migraine development. Whilst further work is needed to fully elucidate these mechanisms, this has promising implications for the clinic, but also for furthering our understanding of migraine pathophysiology. The findings suggest that behavioural interventions aimed at improving sleep and pharmacological targets involving the hypothalamic orexinergic system could potentially lessen the burden for the many patients which suffer with migraine.

Appendices

Appendix 1: von Frey Calculator

Table 1: tabular value for *k* based on the response pattern to calculate the 50% mechanical withdrawal threshold from Chaplan et al. (1994)

Pattern	Value for *k*	Pattern	Value for *k*	Pattern	Value for *k*	Pattern	Value for *k*
OX	−0.5	OOOXOOOO	−0.547	XO	0.5	XXXOXXXX	0.547
OOX	−0.388	OOOOXOOOO	−0.547	XXO	0.388	XXXXOXXXX	0.547
OOOX	−0.378	OXOOOX	−1.25	XXXO	0.378	XOXXXO	1.25
OOOOX	−0.377	OOXOOOX	−1.247	XXXXO	0.377	XXOXXXO	1.247
OXO	0.842	OOOXOOOX	−1.246	XOX	−0.842	XXXOXXXO	1.246
OOXO	0.89	OOOOXOOOX	−1.246	XXOX	−0.89	XXXXOXXXO	1.246
OOOXO	0.894	OXOOXO	0.372	XXXOX	−0.894	XOXXOX	−0.372
OOOOXO	0.894	OOXOOXO	0.38	XXXXOX	−0.894	XXOXXOX	−0.38
OXX	−0.178	OOOXOOXO	0.381	XOO	0.178	XXXOXXOX	−0.381
OOXX	0	OOOOXOOXO	0.381	XXOO	0	XXXXOXXOX	−0.381
OOOXX	0.026	OXOOXX	−0.169	XXXOO	−0.026	XOXXOO	0.169
OOOOXX	0.028	OOXOOXX	−0.144	XXXXOO	−0.028	XXOXXOO	0.144
OXOO	0.299	OOOXOOXX	−0.142	XOXX	−0.299	XXXOXXOO	0.142
OOXOO	0.314	OOOOXOOXX	−0.142	XXOXX	−0.314	XXXXOXXOO	0.142
OOOXOO	0.315	OXOXOO	0.022	XXXOXX	−0.315	XOXOXX	−0.022
OOOOXOO	0.315	OOXOXOO	0.039	XXXXOXX	−0.315	XXOXOXX	−0.039
OXOX	−0.5	OOOXOXOO	0.04	XOXO	0.5	XXXOXOXX	−0.04
OOXOX	−0.439	OOOOXOXOO	0.04	XXOXO	0.439	XXXXOXOXX	−0.04
OOOXOX	−0.432	OXOXOX	−0.5	XXXOXO	0.432	XOXOXO	0.5
OOOOXOX	−0.432	OOXOXOX	−0.458	XXXXOXO	0.432	XXOXOXO	0.458
OXXO	1	OOOXOXOX	−0.453	XOOX	−1	XXXOXOXO	0.453
OOXXO	1.122	OOOOXOXOX	−0.453	XXOOX	−1.122	XXXXOXOXO	0.453
OOOXXO	1.139	OXOXXO	1.169	XXXOOX	−1.139	XOXOOX	−1.169
OOOOXXO	1.14	OOXOXXO	1.237	XXXXOOX	−1.14	XXOXOOX	−1.237
OXXX	0.194	OOOXOXXO	1.247	XOOO	−0.194	XXXOXOOX	−1.247
OOXXX	0.449	OOOOXOXXO	1.248	XXOOO	−0.449	XXXXOXOOX	−1.248
OOOXXX	0.5	OXOXXX	0.611	XXXOOO	−0.5	XOXOOO	−0.611
OOOOXXX	0.506	OOXOXXX	0.732	XXXXOOO	−0.506	XXOXOOO	−0.732
OXOOO	−0.157	OOOXOXXX	0.756	XOXXX	0.157	XXXOXOOO	−0.756
OOXOOO	−0.154	OOOOXOXXX	0.758	XXOXXX	0.154	XXXXOXOOO	−0.758
OOOXOOO	−0.154	OXXOOO	−0.296	XXXOXXX	0.154	XOOXXX	0.296
OOOOXOOO	−0.154	OOXXOOO	−0.266	XXXXOXXX	0.154	XXOOXXX	0.266
OXOOX	−0.878	OOOXXOOO	−0.263	XOXXO	0.878	XXXOOXXX	0.263
OOXOOX	−0.861	OOOOXXOOO	−0.263	XXOXXO	0.861	XXXXOOXXX	0.263
OOOXOOX	−0.86	OXXOOX	−0.831	XXXOXXO	0.86	XOOXXO	0.831
OOOOXOOX	−0.86	OOXXOOX	−0.763	XXXXOXXO	0.86	XXOOXXO	0.763
OXOXO	0.701	OOOXXOOX	−0.753	XOXOX	−0.701	XXXOOXXO	0.753
OOXOXO	0.737	OOOOXXOOX	−0.752	XXOXOX	−0.737	XXXXOOXXO	0.752
OOOXOXO	0.741	OXXOXO	0.831	XXXOXOX	−0.741	XOOXOX	−0.831
OOOOXOXO	0.741	OOXXOXO	0.935	XXXXOXOX	−0.741	XXOOXOX	−0.935
OXOXX	0.084	OOOXXOXO	0.952	XOXOO	−0.084	XXXOOXOX	−0.952
OOXOXX	0.169	OOOOXXOXO	0.954	XXOXOO	−0.169	XXXXOOXOX	−0.954
OOOXOXX	0.181	OXXOXX	0.296	XXXOXOO	−0.181	XOOXOO	−0.296
OOOOXOXX	0.182	OOXXOXX	0.463	XXXXOXOO	−0.182	XXOOXOO	−0.463
OXXOO	0.305	OOOXXOXX	0.5	XOOXX	−0.305	XXXOOXOO	−0.5
OOXXOO	0.372	OOOOXXOXX	0.504	XXOOXX	−0.372	XXXXOOXOO	−0.504
OOOXXOO	0.38	OXXXOO	0.5	XXXOOXX	−0.38	XOOOXX	−0.5
OOOOXXOO	0.381	OOXXXOO	0.648	XXXXOOXX	−0.381	XXOOOXX	−0.648
OXXOX	−0.305	OOOXXXOO	0.678	XOOXO	0.305	XXXOOOXX	−0.678
OOXXOX	−0.169	OOOOXXXOO	0.681	XXOOXO	0.169	XXXXOOOXX	−0.681
OOOXXOX	−0.144	OXXXOX	−0.043	XXXOOXO	0.144	XOOOXO	0.043
OOOOXXOX	−0.142	OOXXXOX	0.187	XXXXOOXO	0.142	XXOOOXO	−0.187
OXXXO	1.288	OOOXXXOX	0.244	XOOOX	−1.288	XXXOOOXO	−0.244
OOXXXO	1.5	OOOOXXXOX	0.252	XXOOOX	−1.5	XXXXOOOXO	−0.252
OOOXXXO	1.544	OXXXXO	1.603	XXXOOOX	−1.544	XOOOOX	−1.603
OOOOXXXO	1.549	OOXXXXO	1.917	XXXXOOOX	−1.549	XXOOOOX	−1.917
OXXXX	0.555	OOOXXXXO	2	XOOOO	−0.555	XXXOOOOX	−2
OOXXXX	0.897	OOOOXXXXO	2.014	XXOOOO	−0.897	XXXXOOOOX	−2.014
OOOXXXX	0.985	OXXXXX	0.893	XXXOOOO	−0.985	XOOOOO	−0.983
OOOOXXXX	1	OOXXXXX	1.329	XXXXOOOO	−1	XXOOOOO	−1.329
OXOOOO	−0.547	OOOXXXXX	1.465	XOXXXX	0.547	XXXOOOOO	−1.465
OOXOOOO	−0.547	OOOOXXXXX	1.496	XXOXXXX	0.547	XXXXOOOOO	−1.496

Appendix 2: PRISMA Checklist

	Item #	Checklist item	Location where item is reported
TITLE			
Title	1	Identify the report as a systematic review and/or meta-analysis.	107
ABSTRACT			
Abstract	2	See the PRISMA 2020 for Abstracts checklist.	165
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of existing knowledge.	106-108
Objectives	4	Provide an explicit statement of the objective(s) or question(s) the review addresses.	109
METHODS			
Eligibility criteria	5	Specify the inclusion and exclusion criteria for the review and how studies were grouped for the syntheses.	110
Information sources	6	Specify all databases, registers, websites, organisations, reference lists and other sources searched or consulted to identify studies. Specify the date when each source was last searched or consulted.	109-110
Search strategy	7	Present the full search strategies for all databases, registers and websites, including any filters and limits used.	109, 111
Selection process	8	Specify the methods used to decide whether a study met the inclusion criteria of the review, including how many reviewers screened each record and each report retrieved, whether they worked independently, and if applicable, details of automation tools used in the process.	109, 110, 112
Data collection process	9	Specify the methods used to collect data from reports, including how many reviewers collected data from each report, whether they worked independently, any processes for obtaining or confirming data from study investigators, and if applicable, details of automation tools used in the process.	113-114
Data items	10a	List and define all outcomes for which data were sought. Specify whether all results that were compatible with each outcome domain in each study were sought (e.g. for all measures, time points, analyses), and if not, the methods used to decide which results to collect.	112-113
	10b	List and define all other variables for which data were sought (e.g. participant and intervention characteristics, funding sources). Describe any assumptions made about any missing or unclear information.	112-113
Study risk of bias assessment	11	Specify the methods used to assess risk of bias in the included studies, including details of the tool(s) used, how many reviewers assessed each study and whether they worked independently, and if applicable, details of automation tools used in the process.	114-116
Effect measures	12	Specify for each outcome the effect measure(s) (e.g. risk ratio, mean difference) used in the synthesis or presentation of results.	114
Synthesis methods	13a	Describe the processes used to decide which studies were eligible for each synthesis (e.g. tabulating the study intervention characteristics and comparing against the planned groups for each synthesis (item #5)).	114
	13b	Describe any methods required to prepare the data for presentation or synthesis, such as handling of missing summary statistics, or data conversions.	114
	13c	Describe any methods used to tabulate or visually display results of individual studies and syntheses.	114
	13d	Describe any methods used to synthesize results and provide a rationale for the choice(s). If meta-analysis was performed, describe the model(s), method(s) to identify the presence and extent of statistical heterogeneity, and software package(s) used.	114-115
	13e	Describe any methods used to explore possible causes of heterogeneity	115

	Item #	Checklist item	Location where item is reported
		among study results (e.g. subgroup analysis, meta-regression).	
	13f	Describe any sensitivity analyses conducted to assess robustness of the synthesized results.	115
Reporting bias assessment	14	Describe any methods used to assess risk of bias due to missing results in a synthesis (arising from reporting biases).	114-115
Certainty assessment	15	Describe any methods used to assess certainty (or confidence) in the body of evidence for an outcome.	116
RESULTS			
Study selection	16a	Describe the results of the search and selection process, from the number of records identified in the search to the number of studies included in the review, ideally using a flow diagram.	116-117, Figure 23
	16b	Cite studies that might appear to meet the inclusion criteria, but which were excluded, and explain why they were excluded.	117
Study characteristics	17	Cite each included study and present its characteristics.	Table 8
Risk of bias in studies	18	Present assessments of risk of bias for each included study.	122
Results of individual studies	19	For all outcomes, present, for each study: (a) summary statistics for each group (where appropriate) and (b) an effect estimate and its precision (e.g. confidence/credible interval), ideally using structured tables or plots.	Figures 25, 26, Tables 13 & 14
Results of syntheses	20a	For each synthesis, briefly summarise the characteristics and risk of bias among contributing studies.	112
	20b	Present results of all statistical syntheses conducted. If meta-analysis was done, present for each the summary estimate and its precision (e.g. confidence/credible interval) and measures of statistical heterogeneity. If comparing groups, describe the direction of the effect.	124-133
	20c	Present results of all investigations of possible causes of heterogeneity among study results.	124-133
	20d	Present results of all sensitivity analyses conducted to assess the robustness of the synthesized results.	124-133
Reporting biases	21	Present assessments of risk of bias due to missing results (arising from reporting biases) for each synthesis assessed.	Figure 24, 122
Certainty of evidence	22	Present assessments of certainty (or confidence) in the body of evidence for each outcome assessed.	124-126
DISCUSSION			
Discussion	23a	Provide a general interpretation of the results in the context of other evidence.	134-137
	23b	Discuss any limitations of the evidence included in the review.	137-143
	23c	Discuss any limitations of the review processes used.	143-144
	23d	Discuss implications of the results for practice, policy, and future research.	143
OTHER INFORMATION			
Registration and protocol	24a	Provide registration information for the review, including register name and registration number, or state that the review was not registered.	109
	24b	Indicate where the review protocol can be accessed, or state that a protocol was not prepared.	109
	24c	Describe and explain any amendments to information provided at registration or in the protocol.	N/A
Support	25	Describe sources of financial or non-financial support for the review, and the role of the funders or sponsors in the review.	N/A
Competing	26	Declare any competing interests of review authors.	N/A

	Item #	Checklist item	Location where item is reported
interests			
Availability of data, code and other materials	27	Report which of the following are publicly available and where they can be found: template data collection forms; data extracted from included studies; data used for all analyses; analytic code; any other materials used in the review.	N/A

Appendix 3: Adapted Newcastle - Ottawa Quality Assessment Scale Case Control Studies

<u>Note</u>: A study can be awarded a maximum of one star for each numbered item within the Selection category. A maximum of two stars can be given for Comparability.

Selection (maximum 4 points)

1) <u>Is the case definition of migraine adequate</u>?

    a) yes, with validation based on ICHD criteria*

    b) yes, eg record linkage or based on self-report

    c) no description

2) <u>Representativeness of the cases</u>

    a) consecutive or obviously representative series of cases*

    b) potential for selection biases or not stated

3) <u>Selection of Controls</u>

    a) community controls*

    b) hospital controls

    c) no description

4) <u>Definition of Controls</u>

    a) no history of migraine (endpoint)*

    b) no description of source

Comparability

1) <u>Comparability of cases and controls on the basis of the design or analysis</u>

a) study controls for age*

b) study controls for any additional factor e.g. sex, BMI*

Appendix 4: Adapted Newcastle - Ottawa Quality Assessment Scale For Cross-Sectional Studies

Note: A study can be awarded a maximum of one star for each numbered item within the Selection category. A maximum of two stars can be given for Comparability.

Selection (maximum 4 points)

1) Representativeness of the sample

   a) Truly representative of the average in the target population (e.g. random sampling)*

   b) Somewhat representative (non-random sampling)

   c) No description of the sampling strategy

2) Sample size

   a) Pre-determined and satisfactory e.g. based on power calculation*

   b) Not pre-calculated

3) Non-respondents

   a) Comparability between respondents and non-respondents is established and/or the response rate is satisfactory*

   b) The response rate is unsatisfactory, or the comparability between responders and non-responders unsatisfactory

   c) No description of the response rate or the characteristics of the responders and non-responders

4) Is the case definition of migraine adequate?

   a) yes, with validation based on ICHD criteria*

b) yes, eg record linkage

c) no description

Comparability (maximum 2 points)

1) <u>Comparability of those with migraine and controls on the basis of the design or analysis</u>

a) study controls for the most important factor (age) (either in the selection or as a variable in the statistical analysis) *

b) study controls for any additional factor e.g. sex

Appendix 5: STROBE Checklist

STROBE Statement—checklist of items that should be included in reports of observational studies.

	Item No.	Recommendation	Page No.
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	N/A
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	147-148
Objectives	3	State specific objectives, including any prespecified hypotheses	148-149
Methods			
Study design	4	Present key elements of study design early in the paper	150
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	150

Participants	6	(*a*) *Cohort study*—Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up	150-151
		Case-control study—Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls	
		Cross-sectional study—Give the eligibility criteria, and the sources and methods of selection of participants	
		(*b*) *Cohort study*—For matched studies, give matching criteria and number of exposed and unexposed	N/A
		Case-control study—For matched studies, give matching criteria and the number of controls per case	
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	152-153
Bias	9	Describe any efforts to address potential sources of bias	N/A
Study size	10	Explain how the study size was arrived at	150-151

Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	154-155
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	154-155
		(*b*) Describe any methods used to examine subgroups and interactions	N/A
		(*c*) Explain how missing data were addressed	N/A
		(*d*) *Cohort study*—If applicable, explain how loss to follow-up was addressed *Case-control study*—If applicable, explain how matching of cases and controls was addressed *Cross-sectional study*—If applicable, describe analytical methods taking account of sampling strategy	N/A
		(*e̲*) Describe any sensitivity analyses	N/A

Results

Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	155-156
		(b) Give reasons for non-participation at each stage	155-156
		(c) Consider use of a flow diagram	N/A

Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	Figures 27-30, Tables 17-18
		(b) Indicate number of participants with missing data for each variable of interest	156
		(c) *Cohort study*—Summarise follow-up time (eg, average and total amount)	N/A
Outcome data	15*	*Cohort study*—Report numbers of outcome events or summary measures over time	N/A
		Case-control study—Report numbers in each exposure category, or summary measures of exposure	N/A
		Cross-sectional study—Report numbers of outcome events or summary measures	Tables 17-18
N/Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	156-166
		(*b*) Report category boundaries when continuous variables were categorized	N/A
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	N/A

Discussion

Key results	18	Summarise key results with reference to study objectives	166
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	169-173
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	174
Generalisability	21	Discuss the generalisability (external validity) of the study results	169
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	N/A

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

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