Investigations of mode of action of single pulse Transcranial Magnetic Stimulation (sTMS) in animal models and effectiveness in migraine patients

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Thesis submitted for the Degree of Doctor of Philosophy at the Institute of Psychiatry, Psychology and Neuroscience, King's College London

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APRIL 26, 2021

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Declaration

I, Joseph Lloyd, confirm that the work in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in this thesis.

Abstract

Migraine is the 6th most common cause of disability worldwide with high socio-economic and personal impacts. More than just a headache, it presents with nausea, vomiting, photophobia and phonophobia. Around 30% of patients also experience aura, a transient neurological disturbance, usually preceding the headache phase of the migraine. The underlying physiology of migraine aura is thought to be a spreading wave of cortical spreading depression (CSD).

Neuromodulation techniques have been suggested to hold some promise in the acute and preventive treatment of migraine. One potential neuromodulation treatment is the single-pulse transcranial magnetic stimulation (sTMS). sTMS is a non-invasive neuromodulation treatment, that uses magnetic pulses across the scalp and skull to induce weak electrical currents in the underlying cortical tissue. sTMS has previously been shown to be a successful clinical treatment for patients with migraine with aura, as an acute treatment producing an effective reduction in pain scores for up to 48 hours post-treatment and as a preventative treatment reducing headache days after use for 3 months. Further post-market analysis studies have shown its efficacy in the prevention of migraine. In experimental migraine models, sTMS has also successfully blocked chemical and mechanically induced cortical CSD in vivo, however, the precise mechanism by which sTMS causes this effect remains unclear.

Results from this study illustrate that acute application sTMS modulated activity of the cortex it was directly applied to. sTMS causes inhibition of spontaneous and glutamate induced cortical neuronal activity. sTMS also alters activity of cortical spreading depression, increasing the threshold of activation and altering properties of the wave once initiated. The observations suggest the modulation of excitatory cortical neurons via the recruitment of inhibitory GABAergic systems. sTMS' effect on glutamate activity and CSD threshold is negated with the use of GABA antagonists. These finding provide a better understanding for how sTMS acts on the cortex to achieve an acute reduction in headache pain. Inhibiting activity within the cortex also has secondary effect on cortically connected brain structures causing reduced activity in the thalamus and hypothalamus. Reducing thalamic activity may disrupt incoming trigeminal spinal signals that drive a migraine attack. These acute effects on the cortex and thalamus accumulate with long term application, with implications for how sTMS is able to act as a preventative treatment. Long term cortical application of sTMS also does not appear to cause

sensitisation of the TCC. This would suggest that prolonged use would not develop into a peripheral sensitisation equivalent to medication overuse headache.

In conclusion, this thesis suggests that sTMS can be an effective preventive treatment for a subset of migraine patients. Its mechanisms of action involve acute and long-term interactions with cortical activity, in particular with GABAergic circuits, as well as direct or indirect interactions with subcortical nuclei that are key in migraine pathophysiology.

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Acknowledgements

There are many people I need to thank for helping to make this thesis a reality. Firstly, I would like to thank my family for their support and guidance, particularly Dr P. Lloyd, Mr A. Lloyd and Miss H. Josephs for their repeated help reading and correcting many unreadable drafts.

I would like to thank my supervisors; Dr A. Andreou and Professor S. McMahon, for all their help and support I am especially grateful. Too many members of the community of labs in the CARD have provided expertise, guidance, and friendship to name individually. But to call out a few, my colleges in the Headache Research Lab; Dr B. Okein, Dr T. Takahashi, and Ms R. Abuukar Abdullahi, Dr M. Jones and Dr T. Sears for their invaluable expertise in electrophysiology and Dr K. Chisholm and Dr B. Oehler for their training in *in vivo* calcium imaging.

Thank you to all the hardworking staff of the Headache centre at Guys and St Thomas' NHS Trust, especially; Ms B. Hill, Ms M. Murphy and Dr G. Lambru for their help providing data and constructive guidance in the clinical aspects of the thesis.

The Migraine Trust for providing the funding that has allowed this research to take place.

Finally, I am thankful for the necessary comfort that has been provided by St. Christopher.

Glossary

5-HT,	5-hydroxytryptamine
ACC,	Anterior cingulate cortex
A/P,	Anterior/posterior
AMPA,	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ATP,	Adenosine triphosphate
BDNF,	Brain derived neurotropic factor
BBB,	Blood brain barrier
BOLD,	Blood oxygen level dependent
BoNT/A,	Onabotulinum toxin type A
BSA,	Bovine Serum Albumin
BSU,	Biological services unit
CCK,	Cholecystokinin
CED,	Cambridge Electronic Design
CGRP,	Calcitonin gene-related peptide
CNS,	Central nervous system
CSD,	Cortical Spreading Depression
CSF,	Cerebrospinal fluid
SCN	Corticospinal neurone
cTBS,	Continuous theta-burst stimulation
D/V,	Dorsal/ventral
DC,	Direct current
DCS,	Direct current stimulation
DLPFC,	Dorsolateral prefrountal cortex
DTI,	Diffusion tensor imaging
EEG,	Electroencephalography
EFNS,	European Federation of Neurological Societies
eGFP,	Enhanced green fluorescence protein
EMA,	European Medicines Agency
ESPOUSE,	eNeura SpringTMS Post-Market Observational U.S. Study of Migraine
FAD,	Flavin adenine dinucleotide
FDA,	United States Food and Drug Administration

FMN,	Flavin mononucleotide
fMRI,	Functional magnetic resonance imaging
FSH,	Follicle-stimulating hormone
GABA,	Gamma Aminobutyric Acid
GHP,	Gamma-Hydroxybutyric acid
GP,	General practitioner
GSTT,	Guy's and St Thomas' NHS Trust
GTN,	Glycerol trinitrate
GWAS,	Genome wide association studies
HF,	High frequency
HF10 SCS,	High frequency (10 Hz) spinal cord stimulation
HFE,	High frequency episodic migraine
HIT-6,	Headache Impact Test 6
HM,	Hemiplegic migraine
HPA,	Hypothalamic-pituitary-adrenal
HIS,	International Headache Society
ICHD,	The International Classification of Headache Disorders
INS,	International Neuromodulation Society
IQ,	Inter quartile range
iTBS,	Intermittent theta-burst stimulation
KCL,	King's College London
LC,	Locus coeruleus
LF,	Low frequency
LTD,	Long-term depression
LTP,	Long-term potentiation
LVA,	Low voltage activated
mAB,	Monoclonal antibodies
M/L,	Medial/lateral
MEP,	Motor evoked potentials
MHD,	Monthly headache days
MMD,	Monthly migraine days
MOH,	Medication overuse headache

MR,	Magnetic resonance
MSPA,	Magnetic suppression of perceptual accuracy
Mw/oA,	Migraine without aura
MwA,	Migraine with aura
NICE,	National Institute of Health and Care Excellence
NIRS,	Near infrared spectroscopy
NMDA,	N-mehyl-D-aspartate
NHS,	National Health Service
NO,	Nitric oxide
NSAIDs,	Non-steroidal anti-inflammatory drugs
OCT,	Optimal cutting temperature
OD,	Outer diameter
OIH,	Opioid-induced hyperalgesia
ONS,	Occipital nerve stimulation
PACAP,	Pituitary adenylate cyclase-activating polypeptide
PAG,	Peri-acqueductal grey
PET,	Positron emission tomography
PBS,	Phosphate buffered saline
PFA,	Paraformaldehyde
ppTMS,	Paired pulsed transcranial magnetic stimulation
PSH,	Post-stimulus histograms
PSMT,	Precision Trinocular Surgical Microscope
RCTs,	Randomised control trials
rmANOVA,	Repeated measures analysis of variance
RNM,	Raphe nucleus magnus
ROI,	Regions of interest
rTMS,	Repeated measures transcranial magnetic stimulation
RVM,	Rosteroventromedial medulla
SD,	Standard deviation
SEM,	Standard error of the mean
SICF	Short interval cortical facilitation
SICI	Short interval cortical inhibition

SMC,	Scottish Medicine Consortium
SNAP-25,	Synaptosomal-associated protein 25
SPECT,	Single-photon emission computed tomography
SPG,	Sphenopalatine ganglion
SPGS,	Sphenopalatine ganglion stimulation
SSS,	Superior sagittal sinus
sTMS,	Single-pulse transcranial magnetic stimulation
SUNA,	Short-lasting unilateral neuralgiform headache attacks with cranial autonomic symptoms
SUNCT,	Short-lasting unilateral neuralgiform headache attacks with conjunctival injection and tearing
TACs,	Trigeminal Autonomic Cephalalgias
TBS,	Theta-burst stimulation
TCC,	Trigeminal cervical complex
tDCS,	Transcranial direct current stimulation
TG,	Trigeminal ganglion
TMN,	Tuberomammillary nucleus
TMS,	Transcranial magnetic stimulation
TNC,	Trigeminal nucleus caudalis
TRF,	Thalamic reticular formation
TTH,	Tension type headache
UV,	Ultra-Violet
VD	Voluntary drive
VIP,	Vasoactive intestinal peptide
VNS,	Vagus nerve stimulation
VPM,	Ventroposteromedial

Chapter 1: Introduction

Chapter 1:
Introduction

Thesis Overview

Neuromodulation is a range of treatment options with great potential in the treatment of migraine. In particular, single pulse Transcranial Magnetic Stimulation (sTMS) has shown great utility as both an acute and preventative migraine treatment. sTMS has advantages over traditional pharmacological treatments by being targeted, localised and without significant side effects.

Via electromagnetic conduction, sTMS generates an electrical current in the cortex directly below the coil. Application of sTMS has been shown to alleviate pain during an attack (Lipton et al., 2010) and long-term application has been shown to cause a reduction in monthly migraine days (Starling et al., 2018).

While sTMS has been shown to be efficacious for migraine treatment there is a knowledge disconnect between the known physics and the observed clinical outcomes. The aim of the studies presented in this thesis is to bridge this gap in understanding. To investigate the direct actions of the migraine sTMS treatment on the cortex, the indirect action on cortically connected subcortical areas, following both acute and long-term application.

sTMS directly interacts with the cortex immediately below the coil, producing an electrical current that can modulate cortical neuronal activity. In the second chapter the aim is to investigate how sTMS directly interacts with the principle cortical features of migraine, namely cortical hyperexcitability and cortical spreading depression (CSD).

The first results chapter will consist of 7 separate experiments exploring the direct effect of sTMS on neuronal activity of the cortex. *(1)* The effect of application of a range of sTMS doses on spontaneous cortical neuronal activity. *(2 & 3)* Investigating application of clinically relevent doses of sTMS on glutamate induced cortical neuronal activity (as a model of cortical hyperexcitability) in isolation and in the presence of GABA antagonists. *(4 & 5)* Investigating the application of sTMS on electrically induced CSD thresholds in isolation and in the presence of GABA antagonists. *(6)* The effect of sTMS application on spontaneous cortical actvity using real time *in vivo* calcium imaging. *(7)* The effect of sTMS application on the properties and progression of a CSD wave using

in vivo calcium imaging. The hypothesis for this chapter is that cortically applied sTMS will directly inhibit cortical neuronal activity, possibly via cortical GABAergic circuits.

Despite only generating an electrical current in the cortex, sTMS has been shown to have substantial effects on cortically connected subcortical nuclei (Andreou et al., 2016). In the third chapter the aim is to investigate how two cortically connected subcortical nuclei: the hypothalamus (active early in a migraine attack and proposed as a possible migraine trigger) and the thalamus (an important relay in ascending trigeminal nociception which become activated during an attack) interact with cortically applied sTMS.

The second result chapters will consist of 4 electrophysiological experiments exploring the indirect effect of sTMS on neuronal activity of the hypothalamus and thalamus. *(1)* Investigating how clinically meaningful doses of sTMS effect thalamic activation thresholds from peripheral electrical stimulation. *(2)* Investigating how CSD effects spontaneous and peripherally evoked thalamic activity. *(3)* The effect of prior sTMS application modulates the previously seen CSD altered sponaneous and peripherally evoked thalamic neuronal activity. *(4)* The effect of sTMS on hypothalamic spontaneous neuronal activity. The hypothesis is that cortically applied sTMS will indirectly alter thalamic and hypothalamic neuronal activity via descending cortical connections.

In addition to being an effective acute treatment for migraine, sTMS has also been shown to be an effective prophylactic treatment for migraine, reducing the prevalance of headache and migraine days over several months of daily treatment. Chapter three aims to investigate if and how, the previously shown acute effects of sTMS accumulate with daily application over a long period.

This will consist of 5 experiments following 30 days of daily sTMS application. *(1)* Investigating the mechanical pain threshold of the trigeminal dermatome, throughout sTMS application, to assess any retrograde effects sTMS may have on peripheral mechanisms. Following the treatment peiod electrophysiological experiments will be carried out to assess the long term effects of sTMS applications. *(2)* The effect of long-term application of sTMS on electrical thresholds for CSD initiation. *(3)* Investigating the effects of long-term application of sTMS on peripherally evoked thalamic activity thresholds. *(4)* The effects of long-term application of sTMS on CSD modulation of spontaneous thalamic activity. The hypothesis is that long-term cortical application of

sTMS will accumulate to alter cortical and subcortical neuronal activity, beyond the acute effects seen.

sTMS has been approved by NICE as a treatment for migraine in the UK. The Headache Centre at Guy's and St Thomas' NHS Trust in London is the first NHS headache clinic to offer sTMS as a commissionable treatment for migraine. The aim of chapter 5 is to conduct prospective post-market clinical audit of sTMS as a migraine prophylactic treatment, in a real world setting with a representitive population of hard-to-treat patients often seen in tertiary healthcare centres.

The primary clinical endpoint will be monthly headache days, with a 30% decrease during the trial period considered to be a responder. Additional measures to evaluate sTMS efficacy will include monthly migraine days, headache-free days, abortive treatment intake days and headache impact test-6 score. Finally, subgroup analysis of additional possible migraine symptoms including; the presence of aura symtoms, previous treatment with Botulinum Toxin type A, medication overuse and presence of vestibular symptoms may shed light on possible subgroups of patients likely to respond most effectively to sTMS treatment.

By combining how sTMS interacts with several areas of the migraine pathway both short and long term, will shed a better understanding of its mechanisms of action in migraine and suggest gaps in the treatment that need to be improved in the future.

In this introductory chapter I will give background to migraines, before discussing the current treatment of migraines. Then I will go on to discuss TMS and Single Pulse TMS, which are the focus of this PhD. In this first section, I will discuss migraine prevalence and cost, the classification of migraines, phases of the migraine attack and migraine pathophysiology.

Chapter 1:

Introduction

Migraine Introduction

Migraine is a common disabling neurological condition, characterised by severe episodic headache attacks with associated neurological symptoms, thought to affect approximately a billion people worldwide (Vos et al., 2017).

Migraine has been described historically for centuries. The ancient Egyptians Ebers papyrus from 1500 BCE, contain a description consistent with migraine. Aretaeus of Cappadocia provided a further description of headaches in the 2nd century (Adams, 1856). The name migraine derives from the term "hemicrania" (ημικρανία) coined by Galen of Pergamon in around 160 AD (Galen, 1890).

Migraine Prevalence and Cost

Migraine is the seventh most disabling disease worldwide and the leading cause of neurological disability (Steiner et al., 2013). Migraine has an estimated prevalence of 14.7%. Migraine predominantly affects females, with approximately 17% of the female population affected as opposed to just 6% of males. According to the latest global burden of disease study published in 2016, migraine is the 6th most prevalent cause of disability worldwide (1.04 billion, ± 0.04 billion), and the second largest cause of years lived with disability (45.1 million) behind lower-back pain (57.6 million) (Vos et al., 2017).

Due to how painful and disabling migraine attacks are, 25 million days are estimated to be lost each year due to absenteeism in the UK alone. This loss of productivity is calculated to cost the UK economy £2.25 billion a year (Steiner et al., 2003). On any given day more than 80,000,000 people are having a migraine attack globally. There is also a significant cost to the NHS, costing £150 million a year in GP visits, and prescription medications (Steiner, 2010). Annually across the whole of Europe, the mean cost of migraine has been calculated to €1222 per-person (Linde et al., 2012).

Migraine Classification

The International Classification of Headache Disorders 3rd edition (ICHD-3) 2018, from the International Headache Society (IHS) classifies migraine as a primary headache

disorder. A primary headache disorder is a condition where the headache is the main symptom of the condition and not a symptom of an underlying condition for example a headache attributed to trauma of the head and/or neck, which is classified as a secondary headache (IHS, 2018)

In addition to migraine there are several other types of primary headache disorders, with the most common being tension type headache and the most painful being Trigeminal Autonomic Cephalalgias (TACs), which include cluster headache, paroxysmal hemicrania, Short-lasting unilateral neuralgiform headache with conjunctival tearing (SUNCT) and Short-lasting unilateral neuralgiform headache (SUNA) (IHS, 2018).

ICHD-3 has the same main division in migraine classification, between migraine with and without aura (Table 1). Migraine without aura (Mw/oA), previously called "common migraine" (ICHD-3, 1.1), is the most prevalent form of migraine (Rasmussen and Olesen, 1992). The ICHD-3 describes Migraine without aura as;

> *"Recurrent headache disorder manifesting in attacks lasting 4-72 hours. Typical characteristics of the headache are unilateral location, pulsating quality, moderate or severe intensity, aggravation by routine physical activity and association with nausea and/or photophobia and phonophobia."* (IHS, 2018)

Migraine with aura (MwA) is less common than migraine without aura affecting ~30% of migraine patients (Stewart et al., 1991). Previously called "classical migraine" (ICHD-3, 1.2), is the migraine as descried above with an additional transient unilateral neurological condition known as migraine aura. In 90% of cases the aura symptoms are visual in nature (Rasmussen and Olesen, 1992), however, can also be somatosensory, aphasia/dysphasia, motor or brainstem related symptoms. Whatever the specific nature of the aura, they follow the same spreading pattern, a brief period of excitation followed by depression (Lashley, 1941).

Table 1: Migraine subtype criteria

1.1 Migraine without aura	
Description	Recurrent headache disorder manifesting in attacks lasting 4-72 hours. Typical characteristics of the headache are unilateral location, pulsating quality, moderate or severe intensity, aggravation by routine physical activity and association with nausea and/or photophobia and phonophobia.
Diagnostic Criteria	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: a. unilateral location b. pulsating quality c. moderate or severe pain intensity d. aggravation by or causing avoidance of routine physical activity (e.g. walking or climbing stairs) D. During headache at least one of the following: a. nausea and/or vomiting b. photophobia and phonophobia E. Not better accounted for by another ICHD-3 diagnosis.
1.2 Migraine with aura	
Description	Recurrent attacks, lasting minutes, of unilateral fully reversible visual, sensory or other central nervous system symptoms that usually develop gradually and are usually followed by headache and associated migraine symptoms.
Diagnostic Criteria	A. At least two attacks fulfilling criteria B and C B. One or more of the following fully reversible aura symptoms: a. visual b. sensory c. speech and/or language d. motor e. brainstem f. retinal C. At least two of the following four characteristics: a. at least one aura symptom spreads gradually over 5 minutes, and/or two or more symptoms occur in succession b. each individual aura symptom lasts 5-60 minutes c. at least one aura symptom is unilateral2 d. the aura is accompanied, or followed within 60 minutes, by headache D. Not better accounted for by another ICHD-3 diagnosis, and transient ischaemic attack has been excluded.
1.3 Chronic migraine	
Description	Headache occurring on 15 or more days per month for more than 3 months, which has the features of migraine headache on at least 8 days per month.
Diagnostic Criteria	A. Headache (tension-type-like and/or migraine-like) on ≥15 days per month for >3 months and fulfilling criteria B and C B. Occurring in a patient who has had at least five attacks fulfilling criteria B-D for 1.1 Migraine without aura and/or criteria B and C for 1.2 Migraine with aura C. On ≥8 days per month for >3 months, fulfilling any of the following 3: 1. criteria C and D for 1.1 Migraine without aura 2. criteria B and C for 1.2 Migraine with aura 3. believed by the patient to be migraine at onset and relieved by a triptan or ergot derivative D. Not better accounted for by another ICHD-3 diagnosis.

Chapter 1:
Introduction

A hemiplegic migraine (HM; ICHD-3, 1.2.3) is a further subclassification of Migraine with Aura that is accompanied by motor weakness, commonly affecting one side of the body. This aura can last for up to 72 hours, although in some cases it can persist for weeks (IHS, 2018). Within this group is the familial hemiplegic migraine which has strong heritability within first- and second-degree relatives (Thomsen et al., 2003), indicating a strong genetic component. This is thought to have some implications for our understanding of the mechanisms of migraine pathophysiology, therefore will be discussed in detail later.

There is debate as to whether Mw/oA and MwA are etiologically distinct conditions or if migraine exists on a severity continuum, with MwA more severe form of Mw/oA (and HM more severe again) (Nyholt et al., 2004) (Ligthart et al., 2006).

Phases of a migraine attack

Premonitory phase

Migraine is more than just the headache (Fig. 2). The earliest phase of a migraine attack, known as the premonitory (or prodrome) phase, has its onset hours to days before the headache occurs. This premonitory phase has several physiological and psychological symptoms including yawning, tiredness, irritability, difficulty concentrating, food cravings, polyuria and sensory sensitivities (Giffin et al., 2003). Improvements in our understanding of the premonitory phase have meant that the reported prevalence is variable across the literature, however a recent study of a tertiary care hospital suggested 90.1% of patients reported at least 1 premonitory symptom (Arif et al., 2019) and a further study found 96.6% (Quintero et al., 2019).

It remains unclear why individual migraine attacks start. As discussed later, a malfunction of the hypothalamic area is a possibility, with the area of the hypothalamus now considered to be part of the “trigger” of a migraine attack. There are a number of risk factors that contribute to a predisposition to attacks including; sex, genetic pre-distribution, obesity, stress and depression (Fig. 1) (May and Schulte, 2016). The idea of external migraine triggers including; bright lights, chocolate and alcohol have been proposed. However, these triggers have been difficult to replicate in an experimental setting. One emerging theory suggests that since the factors suggested to cause the

migraine attacks are proposed by the patients themselves, there are causality issues. The attack may have already begun, and the "triggers" may in fact be misidentified premonitory phase symptoms e.g. a reported chocolate trigger might be a sugar craving. Despite this, the premonitory symptoms can be a useful tool in recognising an impending attack and treating promptly (Schulte et al., 2015).

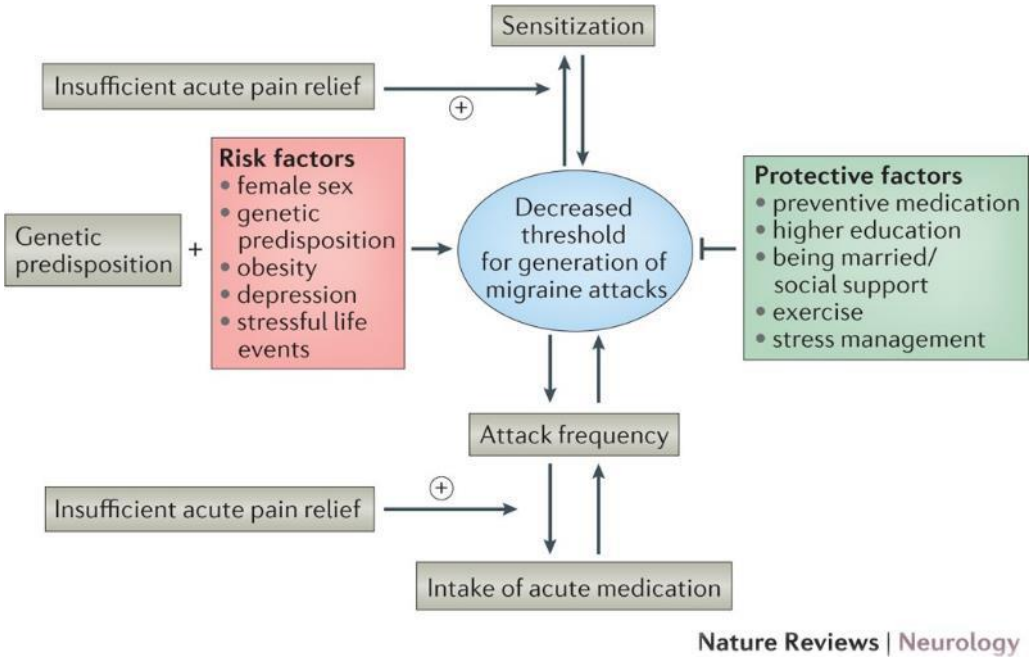

Figure 1: Genetic and nongenetic risk factors contribute to the threshold for the generation of migraine attack*. Insufficient acute pain relief leads to sensitization, which can further lower migraine attack threshold. Increased migraine attack frequency itself also lowers attack threshold; moreover, it increases the intake of acute medication, which can decrease the efficacy of acute pain relief and further predispose to migraine chronification. By contrast, preventive medication and protective factors related to behaviour and lifestyle heighten the threshold and thereby inhibit migraine chronification. (May and Schulte, 2016)*

Migraine aura

In the ~30% of patients that experience aura (Stewart et al., 1991), it occurs in the transition between the premonitory and headache phases, with some overlap between each phases. Although in some cases the onset of migraine aura can be concurrent with the main headache attack. Migraine aura is believed to be caused by a slow depolarising wave known as a Cortical Spreading Depression (CSD), the pathophysiology of which will be covered in more detail in chapter 2 (Acute direct effects of sTMS application on Cortical Neuronal Activity). In 90% of cases the aura is visual, typically presenting as an

arc expanding from the periphery of the vision. This shape is often described as black and white, with scintillating fortification spectra which gives way to a scotoma as it grows larger. In 1941 Lashley illustrated his own visual aura, which described this pattern, showing the gradual development of the symmetrical shape and suggested a cortical origin for the symptoms. The other less common aura types include sensory, language and motor symptoms (motor weakness auras are separately sub-categorised as hemiplegic migraine). Typically, the aura lasts for around an hour.

Headache phase

Following premonitory and aura the main headache phase occurs. This is a throbbing, (usually) unilateral headache that lasts between 4 and 72 hours. The headache pain is also accompanied by additional symptoms including nausea, vomiting, and sensory sensitivities including photophobia, phonophobia, osmophobia and allodynia of the head and scalp (IHS, 2018). This phase is often reported as being the most debilitating, by patients, and produces the symptoms that most patients would like to see addressed. What manifests the headache phase, as discussed below, appears to be abnormal sensory processing along the ascending trigeminothalamic pathway and brainstem descending control.

Postdrome phase

The final phase before the patient completely returns to normal, is the postdrome phase, a hangover of sorts, following the attack phase. The postdrome phase is relatively recently described and the least studied of the migraine attack phases. 81-94% of patients have reported having postdrome symptoms. Average duration of this phase varies between 18 – 25 hours (Kelman, 2006); interestingly the duration of the postdrome phase seems to have no relationship with the severity of the headache attack or any medication taken (Giffin et al., 2016). This phase has similar symptoms to the premonitory phase including fatigue, malaise, difficulty concentrating and neck stiffness. Given the similarities in symptoms, shared networks have been hypothesised between the premonitory and postdrome phase, however there are significant differences in the brain process involved in addition to a near global reduction in cerebral blood flow (Bose et al., 2017). During

the postdrome phase, only the visual cortex (Brodmann areas 17 and 18) became activated more strongly than during the ictal phase (Schulte and May, 2016).

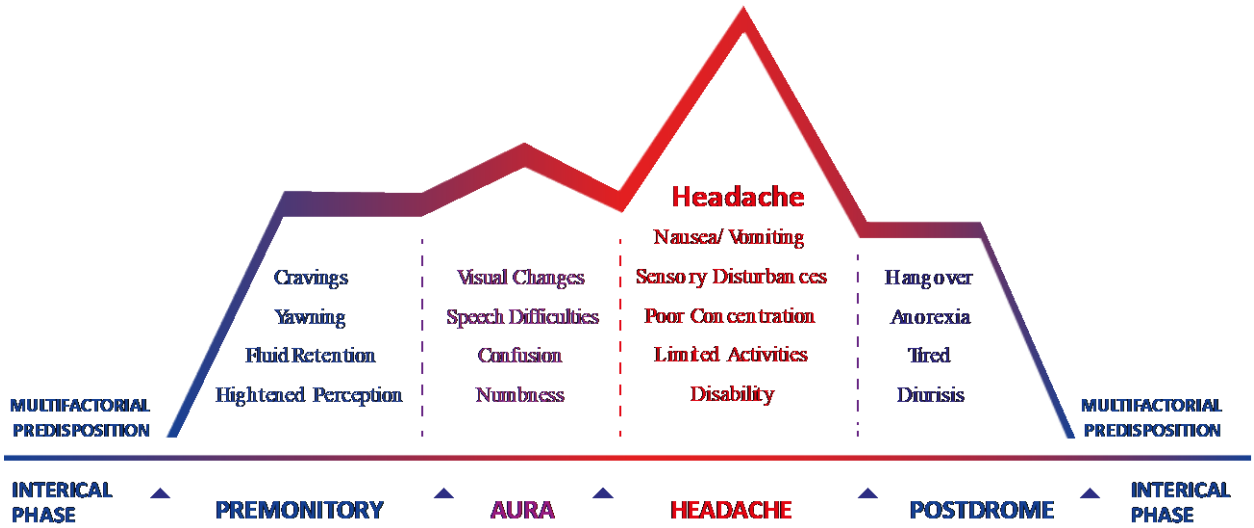

Figure 2: Phases of migraine attack (adapted from Blau,1992)

Migraine Pathophysiology

As opposed to other pain conditions, such as arthritis or chemotherapy induced peripheral neuropathy, migraine attacks do not appear to be directly driven by an external noxious peripheral input, but rather an intrinsic dysfunction of the sensory pathways may be the key player in migraine pathophysiology. Due to the lack of a peripheral trigger, activation along the peripheral nociceptive pathway is most likely driven by (rather than drive) central mechanisms.

During the 20th century the prevalent theory was that migraine originated from the dilation of cranial blood vessels. Initially this theory seems to have credence: the rhythmic throbbing headache certainly points to the involvement of the heartbeat, infusion with vasoactive peptides in migraine patients that do not cross the blood brain barrier (eg calcitonin gene-related peptide (CGRP)), can produce a migraineous-like headache, as can electrical stimulation of dural vasculature, and finally, the treatments available at the time, such as ergotamine caused vasoconstriction. However, the vascular theory of migraine was ultimately dismissed, the frequency of headache pulses was shown not to

synchronise with heat rate, substances such as vasoactive intestinal peptide (VIP), have been shown to cause vasodilation without triggering migraine attack, and the vascular changes were shown to be unrelated to the phase of the attack. So, while the vasculature does play a role in migraine attacks, the purely vascular theory has now largely been debunked, to make way for the more holistic neurovascular theory.

Hypothalamus

The hypothalamus is a small structure below the thalamus at the base of the brain. It has an important role in the control of homeostasis via neuroendocrine connections to the pituitary gland. Particularly it plays an important role for several autonomic functions including circadian rhythms and the stress response (Settle, 2000). The hypothalamus is believed to be involved in migraine pathophysiology, particularly during the premonitory phase where it has been shown to be activated in the first 4 hours of spontaneous migraine attacks (Denuelle et al., 2007).

Hypothalamic role in migraine will be discussed in greater detail in Chapter 3: Acute indirect effects of sTMS application on Thalamic and Hypothalamic Neuronal activity.

Primary trigeminal nerve fibres

During a migraine attack pain is usually perceived as a unilateral throbbing pain in the forehead of moderate to severe intensity (IHS, 2018). The head and face are innervated by the fifth (V) and largest of the cranial nerves the trigeminal nerve, which arises from neurons that have their cell bodies in the trigeminal ganglion. The trigeminal nerve is the main facial sensory nerve, responsible for sensation of innocuous somatosensory stimuli including touch, vibration and pressure, as well as noxious stimuli including pain, temperature and itch.

The trigeminal nerve contains three branches; the ophthalmic (V1), maxillary (V2) and mandibular (V3) branches. The ophthalmic (V1) branch of the trigeminal nerve innervates the ethmoid and sphenoid sinuses, extra and intercranial vasculature (including the middle meningeal artery and the cerebral sinuses) and the dura mater, as well as the skin of the upper portion of the face including the nose, eyes and forehead (Shankland,

2001a). The V1 branch of the trigeminal nerve is thought to be responsible for the transmission of nociceptive inputs during a migraine attack. The V2 branch innervates the lower eyelid and cheek, the nares and upper lip, the upper teeth and gums, , the palate and roof of the pharynx, the nasal mucosa, the maxillary, ethmoid and sphenoid sinuses and some of the meninges (Shankland, 2001b). Finally, the V3 branch innervates parts of the external ear, the lower lip, the lower teeth and gums, the chin and jaw, and parts of the meninges (Shankland, 2001c).

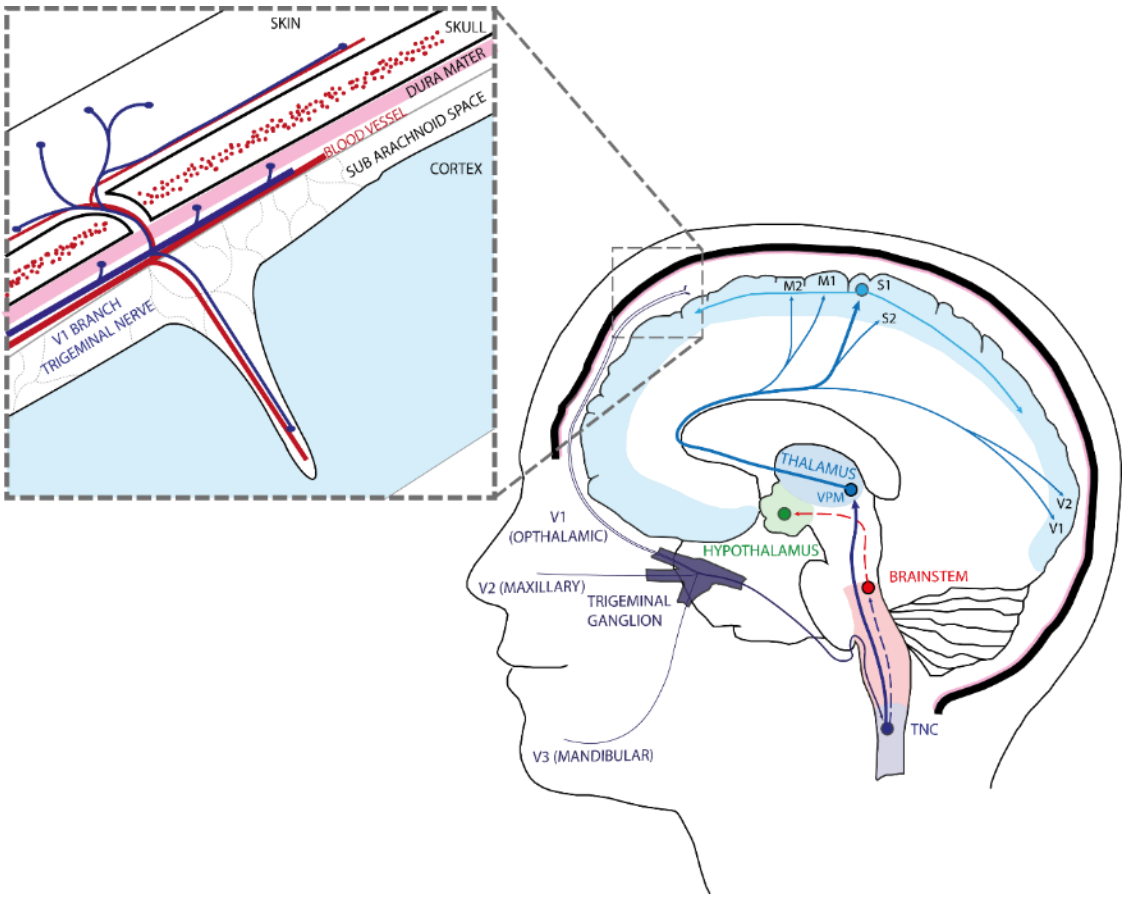

Figure 3: Ascending trigeminal pathways (blue) associated with migraine attacks. *First order neurons of the V1 branch of trigeminal nerve innervate the dura matter, inter and extracranial blood vessels and skin. Synapsing with second order neurones in the dorsal horn of the trigeminal nucleus caudalis (TNC) and ascending to the ventroposteromedial thalamus. Third order neurones ascend to the cerebral cortex.*

Noxious heat, chemical and mechanical stimuli are detected by the peripheral terminals of medium diameter, myelinated Aδ-fibre nociceptors and small diameter, unmyelinated C-fibre nociceptors (McMahon et al., 2013).

Trigeminal ganglion neurones express several peptides including; CGRP, pituitary adenylate cyclase-activating peptide (PACAP), Substance P, vasoactive intestinal peptide (VIP) (Goadsby et al., 1988, Eftekhari et al., 2015). The release of vasoactive neuropeptides, particularly CGRP and substance P from trigeminal first order neurons results in neurogenic sterile inflammation, producing vasodilation, plasma extravasation secondary to capillary leakage, oedema, and mast cell degranulation (Goadsby et al., 1988) (Ottosson and Edvinsson, 1997), further driving trigeminal nociception (Levy et al., 2007).

Among the peptides expressed by the trigeminal ganglion, CGRP is thought to be substantially involved in migraine pathophysiology. Increased levels of CGRP were found in plasma during a migraine attack, while correlate to the timing and severity of the attack (Juhasz et al., 2003). Infused into migraineous patients, CGRP can induce attacks. CGRP dilates extra- and intracerebral arteries (Asghar et al., 2011), and causes an initial non-migraineous headache, as well as a later (>1 hour) migraine-like headache in migraine patients (Lassen et al., 2002) (Hansen et al., 2010). Successful sumatriptan treatment decreases CGRP plasma concentration (Juhasz et al., 2005). While GCRP is a potent vasodilator, this not believed to be its action in migraine. Infusion of the similarly vasoactive neuropeptide VIP, dilates cranial arteries, induces a mild non-migraineous immediate headache but does not provoke migraine attacks (Rahmann et al., 2008). Substance P produces sterile neurogenic inflammation in the dura mater, however substance P antagonists are not effective in the treatment of migraine attacks (Diener, 2003).

Understanding the importance of CGRP in migraine has now led to the development of new migraine therapeutics, including CGRP antagonists and CGRP monoclonal antibodies, which will be discussed in more detail later.

Trigeminocervical complex and spinothalamic afferents

Trigeminal sensory signals travel along the fibres of the trigeminal nerve into the trigeminal cervical complex (TCC), a region of the top of the spinal cord that included the trigeminal nucleus caudalis and the C1 and C2 vertebrae (Fig. 3). Fibres from first order trigeminal neurons enter the CNS at the level of the medulla before travelling

caudally to the level of the C2 vertebra where they synapse with second order neurons, either directly in the superficial layers or via interneurons to the deeper laminae. Stimulation of the trigeminal fibres that innervate the dural vasculature (including superior sagittal, transverse sinuses and the middle meningeal artery), produces increased Fos expression (a marker of cellular activation) in laminae I, II, V, and VI of the TCC.

Three types of second order neurons are found in the dorsal horn of the spinal cord; nociceptive specific neurons in laminae I and II receive nociceptive input from Aδ and C fibres, low threshold neurons found in laminae III and IV receive somatosensory inputs from Aβ fibres, and wide dynamic range neurons in laminae V and VI receive both innocuous and nociceptive inputs from Aβ, Aδ and C fibres. These synapses are believed to be α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-methyl-D-aspartate (NMDA) glutamatergic receptors (McMahon et al., 2013).

The TCC also receives input from the greater occipital nerve. These convergent inputs play an important role in the autonomic symptoms associated with trigeminal autonomic encephalagias as well as neuromodulation treatment techniques.

Thalamocortical afferents

The thalamus is an important relay centre of the ascending trigeminothalamic pathway, collating ascending trigeminal nociceptive signals, and disseminating those signals onwards to higher cortical areas. The ventroposteromedial (VPM) thalamus, is the principal relay nucleus in migraine, relaying information from second order neurons of the TCC to cortical areas involved in the “pain-matrix”, particularly the S1, S2 and insula cortices (Zagami and Lambert, 1990, Zagami and Lambert, 1991).

The role of thalamocortical afferents in migraine will be discussed in greater detail in Chapter 3: Acute indirect effects of sTMS application on Thalamic and Hypothalamic Neuronal activity.

Peripheral and central sensitisation

Repeated activation of peripheral nociceptors can result in them become sensitised, responding with greater intensity to noxious stimuli. This occurs in response to chemical mediators, released from both the nociceptors themselves and non-neuronal cells (including mast cells, platelets, macrophages, neutrophils, fibroblasts, endothelial cells, etc) at the site of injury or inflammation, that act on the nociceptor to enhance its activity (Gangadharan and Kuner, 2013). In migraine pathophysiology, a neurogenic 'sterile' inflammation of the intercranial meninges that drives peripheral sensitisation (Ramachandran, 2018). A wide variety of sensitising mediators are involved with peripheral sensitisation, including protons, adenosine triphosphate (ATP)., prostaglandins, neurotropins, cytokines, chemokines, neuropeptides (such as; CGRP, substance P, bradykinin and histamine) and lipids (Fig. 4) (Gangadharan and Kuner, 2013).

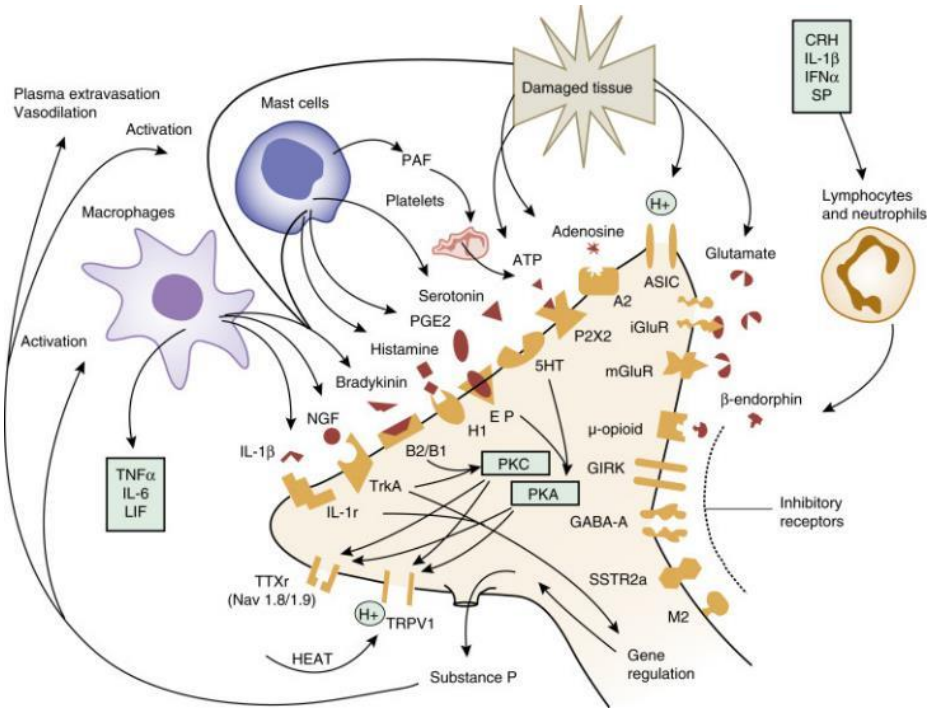

Figure 4: Schematic diagram of the neurochemistry of somatosensory processing at peripheral sensory nerve endings. *5HT, Serotonin receptor; A2, adenosine 2 receptor; ASIC, acid-sensing ion channels; ATP, adenosine triphosphate; B2/B1, bradykinin 2/1 receptors; CRH, corticotropin-releasing hormone; EP, eiconsanoid receptor; GABA, gamma amino butyric acid; GIRK, G-protein coupled inward rectifying potassium channel; H1, histamine 1 receptor; IFN, interferon; iGluR, iontotropic glutamate receptors; IL, interleukin; LIF, leukemia inhibitory factor; M2, muscarinic 2 receptor; mGluR, metabotropic glutamate receptor; NGF, nerve growth factor; P2X2, ATP activated ion channels; PAF, platelet activating factor; PGE2, prostaglandin E2; PKA, protein kinase A; PKC, protein kinase C; SSTR, somatostatin receptor; TNF, tumor necrosis factor; TrkA, tropomyosin receptor kinase A; TRPV1, transient receptor potential vanilloid 1; TTXr, tetrodotoxin resistant (channel).(Nouri et al., 2018)*

As with the first order neurons, the central neurons can also become sensitised. This central sensitisation is characterized by increased excitability, increased synaptic strength and enlargement of the receptive fields (McMahon et al., 1993, Woolf and Doubell, 1994). The converging innocuous and nociceptive inputs onto wide dynamic range second order neurons, mean that once sensitised a painful response can be elicited from a previously innocuous stimulus. This has been hypothesised as the basis for cutaneous allodynia (painful response to an innocuous stimuli) of the trigeminal V1 dermatome (Fig. 5). Additionally, the converging inputs from the three branches of the trigeminal nerve into the TCC can provoke widespread cutaneous hyperalgesia and allodynia of the unilateral side of the head and neck. This mechanism is believed to play a role in the sensitisation of migraine (Dodick and Silberstein, 2006).

NMDA ionotropic glutamate receptor are involved with the induction and maintenance of central sensitisation (Woolf and Thompson, 1991). High frequency trigeminothalamic firing releases, repeatedly depolarised the neurone which removes the magnesium and zinc plugs from the NMDA receptor, allowing sodium influx and potassium efflux and depolarising the neuron (MacDermott and Dale, 1987).

Thalamic neurons are additionally thought to become sensitized to trigeminal stimuli. This has been proposed to contribute to cutaneous allodynia, observed in the majority of migraine patients during attacks (Burstein et al., 1998). Chemical stimulation of the dural trigeminal fibres in animal models has been able to produce sensitisation of the posterior thalamus, with previously innocuous stimuli producing noxious firing patterns (i.e. allodynia) (Burstein et al., 2010).

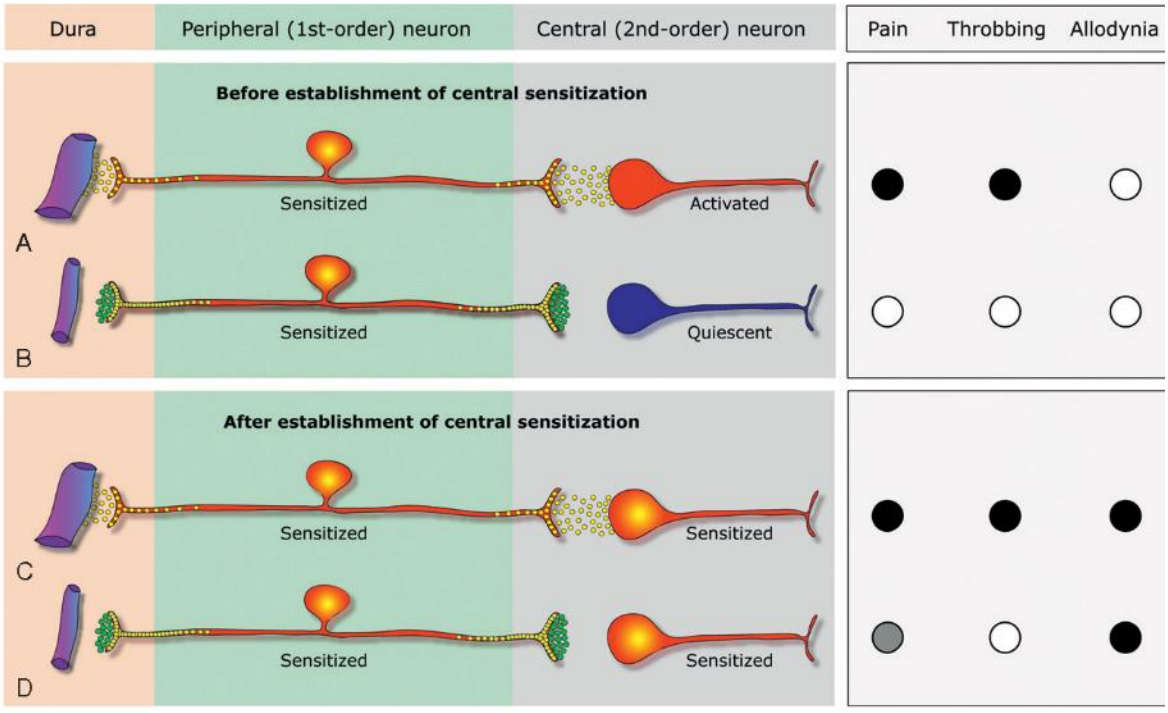

Figure 5: Illustration of neuronal firing of first and second order neurones during peripheral and central sensitisation and the relationship with allodynia and hyperalgesia (Burstein and Jakubowski, 2010)

Cerebral cortex

The cerebral cortex plays a particularly important role in the pathophysiology of migraine. As well as the processing of afferent nociceptive signals from the trigeminal nerve in the somatosensory cortex, migraine aura is believed to be a purely cortical event called cortical spreading depression (CSD). Although migraine was previously believed to be a purely vascular disorder (Wolff, 1948), it is now considered to be primarily a dysfunction of the CNS with aberrant peripheral input (Parsons and Strijbos, 2003).

Further details of the structure and role of the cerebral cortex, including further discussion of cortical spreading depressions and cortical hyperexcitability can be found in Chapter 2: Acute direct effects of sTMS application on cortical neuronal activity.

Corticothalamic Efferents

Interaction between the thalamus and the cortex is not a unidirectional process, there is a dynamic feedback loop back and forth. The cortex provides a great deal of input to the

thalamus, with 10x as many descending corticothalamic axons as ascending thalamocortical projections (Deschênes et al., 1998) modulating thalamic activity and controlling the ascending flow of information.

The role of corticothalamic efferents in migraine will be discussed in greater detail in Chapter 3: Acute indirect effects of sTMS application on Thalamic and Hypothalamic Neuronal activity.

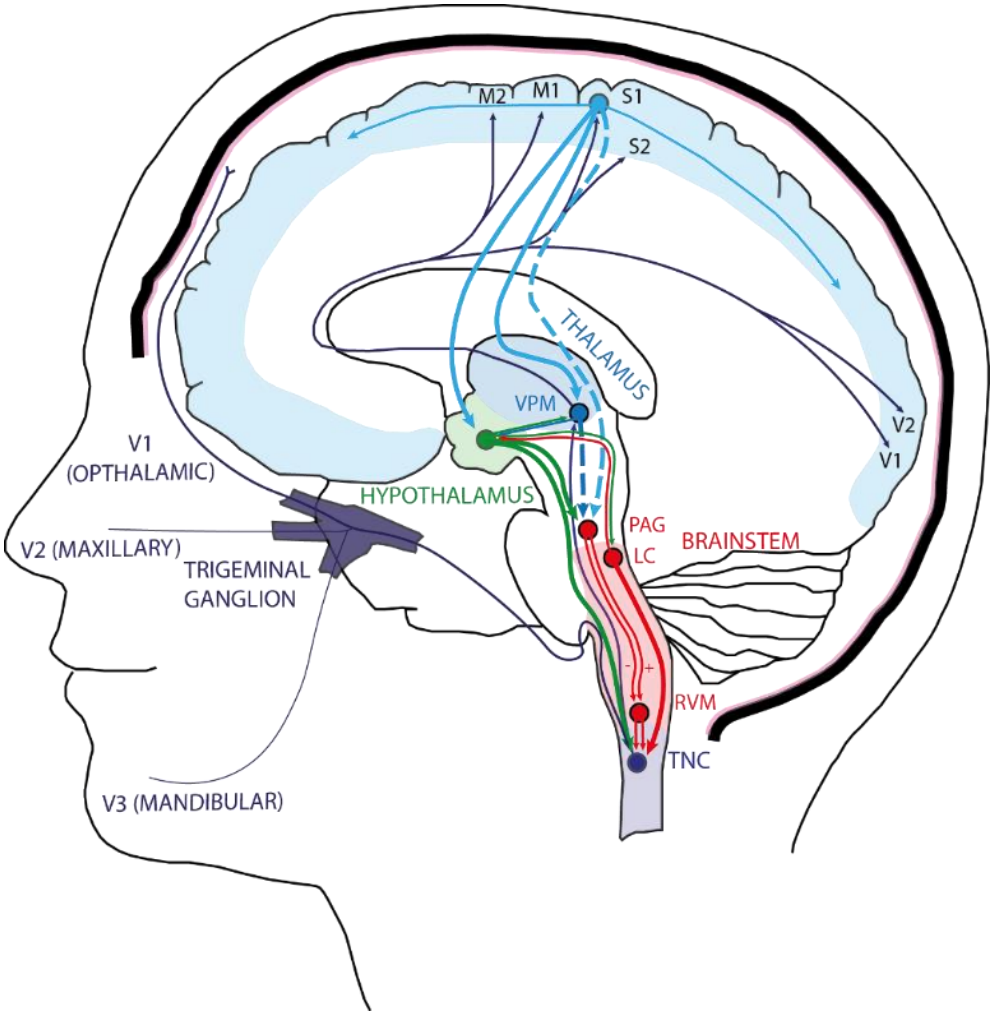

Figure 6: Descending modulating pathways of the of the trigeminothalamic pathways. *Cortical efferent pathways project to the thalamus, hypothalamus, and brainstem. the periaquiductal grey (PAG) recieves input from the cortex, thalamus, hypothalamus and modulates activity of the syanapse between first and secodn order trigeminal neurones in the trigeminal nucleus caudalis (TNC) through the and rostral ventral medulla (RVM). The locus correleus (LC) also modulates activity of the dorsal horn of the spinal cord and has projections to the hypothalamus.*

Brainstem descending efferent modulation

The brainstem, consisting of the medulla oblongata, the pons and the midbrain, lies at the boundary between the spinal cord and the diencephalon. Descending pathways from the brain stem to the spinal cord are known to play an important role in modulating the ascending nociceptive signals (Fig. 6). Activity at the synapse between the first order and second order neurons can be inhibited or facilitated by efferent projections arising from brainstem and midbrain descending efferents. Under normal conditions there is tonic inhibition of nociceptive signals, however in chronic pain states (including migraine) descending facilitation is seen (McMahon et al., 2013). Activation of the brainstem of migraineous patients has been seen in spontaneous (Weiller et al., 1995, Stankewitz and May, 2011) and induced migraine attacks (Afridi et al., 2005b, Bahra et al., 2001), in patients with and without aura as well as both episodic and chronic. However, due to the spatial resolution of the available technology, specific nuclei could not be identified.

The periaqueductal grey (PAG) is a midbrain structure involved with the descending endogenous modulation of spinal nociceptive inputs. Implantation and stimulation of an electrode into the PAG was shown to produce a migraineous-like headache, which was resolved with dihydroergotamine (Raskin et al., 1987), similarly PAG lesions caused by multiple sclerosis are associated with an increase likelihood of migraine-like headaches (Haas et al., 1993, Tortorella et al., 2006, Gee et al., 2005), suggesting the PAG's role in migraine. Several further studies subsequently found that electrical stimulation (Knight and Goadsby, 2001), as well as, $GABA_A$ antagonism (Knight et al., 2003) of the PAG inhibits trigeminal neuronal activity in the TNC. Conversely, blockage of P/Q type calcium channels in the PAG can facilitate trigeminal activity (Bartsch et al., 2005), this suggests the PAG provides tonic inhibition of the dorsal horn of the TNC.

The PAG exerts modulatory control over the TNC predominantly via connection with the rosteroventromedial medulla (RVM). The RVM in conjunction with the raphe nucleus magnus (RNM), provides the primary brainstem structure exerting descending modulation of the dorsal horn of the TNC, with high concentrations of endogenous opioidergic signalling. The RVM contains 'ON' cells which facilitate pain and are inhibited by opioids and the tonically active 'OFF' cells that are activated by opioids. Application of inflammatory mediators to the dura mater have been shown to cause an activation of 'on' cells in the RVM, enhancing pain signalling (McMahon et al., 2013).

Brain scans of migraine patients during the interictal period additionally identified the midbrain structure, the nucleus cuneiformis as having reduced activation to nociceptive stimuli compared to healthy controls (Moulton et al., 2008). The nucleus cuneiformis is involved in descending modulation of nociception, exerting control over activity of a number of brain structures including the PAG and RVM (Basbaum and Fields, 1984, Zemlan and Behbehani, 1988).

The locus coeruleus (LC) is the largest noradrenergic brainstem nucleus, with projections to the neocortex, thalamus, hypothalamus, hippocampus, cerebellum, and spinal cord, and receiving inputs from a number of structures including the hypothalamus. The LC modulates several functions including; cognition, stress, arousal and nociception. Increased functional connectivity between the hypothalamus and LC has been shown interictally (Moulton et al., 2014). The LC has been shown to become activated from migraineous triggers (Tassorelli and Joseph, 1995, Ter Horst et al., 2001).

The involvement of the brainstem may also play a role in the chronification of migraine. Volumetric alterations (Bilgiç et al., 2016) and increased activation (Aurora et al., 2007) of the brainstem have been shown in chronic migraine patients.

Brainstem activation of the dorsal pons, has been seen to persist beyond resolution of pain with successful treatment with sumatriptan (Bahra et al., 2001, Afridi et al., 2004) they suggested that this indicated an underlying dysfunction and not a response to pain, and therefore that migraine attacks may arise from asymmetrical brainstem activation. This may be the case, or it may be that the brainstem activates in response to pain, but in a compensatory fashion to modulate dorsal horn activity, persisting beyond the resolution of pain.

Chapter 1:
Introduction

Current treatments for migraine

I will now review the current available treatment options for migraine looking at acute, preventative and neuromodulation treatments. There are several treatment options available for migraine patients to reduce either the symptoms of an individual attack (acute medications) or the frequency that attacks occur (prophylactic medications) (Table 2). The current mainstay is pharmaceutical drug treatments, however recent advances in neuromodulation have shown that direct stimulation of the nervous system is also an effective treatment option.

Acute

Triptans

Triptans are a family of serotonin, 5-hydroxytryptamine (5-HT)$_{1B/1D}$ receptor agonists, specifically for the treatment of migraine. NICE recommends primarily sumatriptan as the triptan of choice, with the remaining six triptan variants (almotriptan, eletriptan, frovatriptan, naratriptan, rizatriptan and zolmitriptan) available as alternatives.

A meta-analysis of 24,089 patients across 53 clinical trials compared the efficacy of six triptans, with no data for frovatriptan. All 7 triptan variants have been shown to be superior to placebo in clinical trials. Sumatriptan (100mg) was used as a baseline that the subsequent triptans were compared against. Sumatriptan baseline saw a 59% improvement in headache score after 2 hours, with 29% showing pain freedom at 2 hours and 20% sustaining pain freedom up to 24 hours. At least one adverse event was experienced in 13% of patients and 6% experienced a CNS related adverse event. Rizatriptan (10mg) and eletriptan (80mg) showed better efficacy than sumatriptan (100mg) for headache score at 2 hours. Lower efficacy was seen with naratriptan (2.5mg), eletriptan (20mg) and frovatriptan (2.5mg). Almotriptan (12.5mg), zolmitriptan (2.5+5mg), as well as, lower doses of eletriptan (40 mg) and rizatriptan (5mg) showed similar efficacy to sumatriptan. (Ferrari et al., 2001).

5-HT$_{1B}$ receptors are found post-synaptically on smooth muscle of arterial blood vessels, and hence triptans can cause vasoconstriction, including vasoconstriction of the middle cerebral artery and basilar artery, increasing blood flow velocity (Nilsson et al., 1999).

However, it is unclear how much of a role this plays in the treatment of migraine as the majority of patients treated with triptan showed no change in blood flow (Limmroth et al., 1996). Furthermore, it is now understood that modification of vascular tone is not necessary for effective migraine treatment (Rahmann et al., 2008). 5-HT_{1D} receptors are found pre-synaptically on peptidergic C-fibre nociceptors, activation of which can block the release of vasoactive peptides, GCRP and substance P, from trigeminal C-fibre neurons (Goadsby and Edvinsson, 1993), preventing activation and sensitisation of first order neurons.

There is some evidence in animal models to suggest that triptans have some central effects. Direct application supressed trigeminally driven activity of the VPM thalamic nucleus (Shields and Goadsby, 2006) and inhibited TNC activity when applied to the vlPAG (Bartsch et al., 2004a). However, it is unclear how much clinical relevance these findings have due to the hydrophilic nature of many of the triptans, they have historically thought to have very low penetrance across the BBB (Humphrey et al., 1991). Additionally, the lipophilicity of the different triptans have shown no correlation to their clinical outcomes (Pascual and Muñoz, 2005). It is generally accepted that the primary site of action for triptans is peripheral. However a recent study suggested that subcutaneous sumatriptan is rapidly up-taken into the rat brain, including structures like the hypothalamus and brainstem as soon as 1-5 minutes post injection (Muzzi et al., 2020).

Additionally, some of the triptan family (sumatriptan, eletriptan, frovatriptan, naratriptan and zolmitriptan) has been also shown to cause agonism of 5-HT_{1F} receptors, found in trigeminal ganglion neurons and the TNC (as well other neuronal sites including the neocortex and hippocampus). Animal studies of 5-HT1F receptor have found; (1) inhibition of capsaicin-induced neuronal activation of the TNC (Mitsikostas et al., 1999), (2) suppression of electrically stimulated second order neuronal firing of the TNC (Shepheard et al., 1999) but no vasoconstrictive effects (Cohen and Schenck, 1999), which can be explained by the absence of 5-HT_{1F} receptors from the vasculature. This has led to the development of the new specific 5-HT_{1F} agonist lasmiditan, which acts on trigeminal neurons without cardiovascular side effects. Phase 3 randomised control trials of lamitidan found increased pain freedom 2 hours after dosing compared to controls

(32.2% vs 15.3%) (Kuca et al., 2018). At time of writing, Lasmiditan has been approved by the FDA in the USA but still in phase III trials in the UK.

Simple Analgesia- Non-steroidal anti-inflammatory drugs, paracetamol, and aspirin

Over-the-counter analgesics such as non-steroidal anti-inflammatory drugs (NSAIDs) and paracetamol are often taken for relief during migraine attacks. NSAIDs are COX_{1+2} inhibitors, preventing the synthesis of prostaglandins, which among other functions cause inflammation and can activate nociceptors. Headache relief has been shown to be comparable between oral doses of 1000mg of aspirin (Diener et al., 2004b), 400 mg ibuprofen (EMSASI et al., 2004) and 50 mg sumatriptan, although sumatriptan was more effective for complete pain freedom. Paired with 10mg metoclopramide, 900 mg of aspirin has a similar efficacy to 100 mg sumatriptan, however patients rated sumatriptan treatment higher (Hornung et al., 1992). Aspirin can also be administered via intra-venous infusion, shown to be a safe and effective acute treatment for migraine attacks (Diener and Group, 1999, Weatherall et al., 2010, Taneri and Petersen-Braun, 1995). While intravenous aspirin is recommended by the European Federation of Neurological Societies (EFNS) for severe migraine attacks (Evers et al., 2009), it is not however, recommended by the NICE guidelines for migraine (NICE, 2019b). Naproxen has been shown to be effective in reducing the severity of headache pain during migraine attacks (Johnson et al., 1985, Andersson et al., 1989, Nestvold et al., 1985) and in combination with sumatriptan, superior to either drug as monotherapies (Brandes et al., 2007, Smith et al., 2005).

Anti-emetics

Anti-emetics, such as metoclopramine and domperidone are prescribed for the relief of the associated nausea and vomiting that effects 60% of migraine patients. As well as being disabling and unpleasant for the patient, it can additionally disrupt oral application of abortive medications. Use of metaclopramine has been shown to aid the absorption of aspirin, and of other drugs, as it increases gastric emptying (Volans, 1978).

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Historical acute migraine treatments

In addition to the currently prescribed therapies, for the sake of completion I will briefly mention ergotamine alkaloids and opioids, which are historical therapies that are no longer commonly prescribed (Kennis et al., 2013).

Ergot alkaloids are non-specific 5-HT_1 agonists, but due to structural similarities to the monoamine neurotransmitters, can also have agonistic actions on dopamine and noradrenaline receptors. The anti-migraineous effects are a combination of vasoconstriction via binding to 5-HT_{1B} receptors on cranial blood vessels and 5-HT_{1D} receptors on trigeminal nociceptors (Silberstein and McCrory, 2003). In high doses ergot alkaloids can cause ergotism, poisoning caused by ergots, which produces convulsive and gangrenous symptoms. While still licenced by NICE as a treatment for cluster headache, the British National Formulary recommends avoiding the prescription of ergot alkaloids (Kennis et al., 2013).

Opioids are considered the "gold standard" for acute pain relief. A family of alkaloids from the opium poppy, bind to the endogenous opioidergic mu (μ), delta (δ) and kappa (κ) receptors widely distributed throughout the entire nervous system, particularly important for nociceptive modulation. Unfortunately, opioids have been shown to have limited efficacy for the treatment of migraine and come with significant side effects (Kelley and Tepper, 2012) and a significant risk of patients developing addiction, dependence and tolerance (Ballantyne and LaForge, 2007). In long-term opioid use causes a paradoxical tactile and thermal hypersensitivity to develop, termed opioid-induced hyperalgesia (OIH) (Heinricher et al., 2001). Therefore, NICE does not recommend opioids as a first line treatment for migraine (Kennis et al., 2013). Despite not being recommended (Tepper, 2012) opioids are still widely prescribed in the US (Friedman et al., 2015, Mazer-Amirshahi et al., 2014) however, steps are being taken to address this (Miller et al., 2020).

Medication overuse headache

One of the major issues with the currently available acute medications for migraine is medication overuse headache (MOH). Paradoxically, by overusing acute medications to control a pre-existing headache, a feedback loop is created where a headache leads to

medication treatment, leading to further headaches (separate from the initial condition), leading to further intake of analgesics, and so on.

Medication overuse headache will be further discussed in Chapter 4: Long term application of sTMS.

Oral Prophylactic treatments for migraine

There are several prophylactic treatment options for migraine patients aiming to prevent the occurrence of migraine attacks. Several prophylactic options are recommended by the NHS as first-line treatments including; beta-blockers (propranolol), tricyclic antidepressants (amitriptyline) and anti-epileptics (topiramate) (Kennis et al., 2013).

Propranolol is a β-adrenergic receptor antagonist, blocking the action of adrenaline and noradrenaline, primary prescribed for cardio vascular disorders (Al-Majed et al., 2017). In migraine it is believed to inhibit peripheral cranial vasodilation (Widerøe and Vigander, 1974), although propranolol has additionally been shown to have action as β_1 adrenoceptor antagonist in the VPM thalamic nucleus (Shields and Goadsby, 2004). Propranolol was shown to be an effective prevention treatment of migraine (Diamond and Medina, 1976) and superior to placebo treatments (Linde and Rossnagel, 2004). Beta-blockers are the first choice for migraine prevention and often used as comparator drugs to compare newer prophylactic treatments.

Topiramate is an anti-epileptic drug that acts primary on voltage gated sodium channels and $GABA_A$ receptors to decrease neuronal excitation (Shank et al., 2000). In the trigeminothalamic pathway topiramate attenuates activity of the kainate glutamate receptor (Andreou and Goadsby, 2011). Preventative treatment with topiramate has been shown to significantly reduce migraine frequency (Storey et al., 2001) its efficacy can be comparable to propranolol (Diener et al., 2004a). Topiramate has a number of associated adverse effects including; paraesthesia, fatigue, anorexia, nausea, taste alteration, and diarrhoea, these are generally mild to moderate in severity (Brandes, 2005) the greatest cause of discontinuation from topiramate was due to the adverse events (23.7%) (Hepp et al., 2014).

Amitriptyline is a tricyclic antidepressant, it has antagonistic actions on a large variety of receptors for serotonin, histamine, acetylcholine, and adrenergic receptors. Amitriptyline prevents the reuptake of serotonin and noradrenaline from the synaptic cleft, increasing their action on post-synaptic neurone. Amitriptyline has been shown to reduce the severity, frequency and duration of migraine attacks (Ziegler et al., 1993), and earlier studies showed it can have a comparable efficacy to propranolol (Ziegler et al., 1987). Poor sleep quality is known to be an exacerbating factor for migraine attacks (Fernández-de-Las-Peñas et al., 2018) Amitriptyline can also aid in improving sleep quality for migraine patients (Duman et al., 2015).

Should the first-line oral medications be counter indicated, there are several alternative oral treatments available shown to be effective for migraine prophylaxis. Effective alternatives include; the angiotensin receptor blocker, Candesartan cilexetil (used for the treatment of hypertension), Sodium valproate (an anti-epileptic medication). In severe cases the calcium channel blocker, Flunarizine, can be prescribed by a specialist. The NICE recommendation is that prophylactic treatments are tried at the full dose for 3 months before deciding efficacy. A 30-50% reduction in severity and frequency of migraine attacks is considered a good outcome (Kennis et al., 2013).

There has been a swell of interest in more ‘natural’ vitamin and supplement treatments for migraine, including; magnesium, riboflavin and coenzyme Q10. Some studies have shown low magnesium in the brain during migraine attacks (Ramadan et al., 1989), two studies have found magnesium to be an effective treatment (Facchinetti et al., 1991, Peikert et al., 1996) and a further study found it ineffective (Pfaffenrath et al., 1996). Coenzyme Q10 is found primarily in the mitochondria of eukaryotic cells, a component of the electron transport change it participates in the generation of ATP. Migraine has been suggested to be due to disfunction of mitochondrial cells (Yorns and Hardison, 2013). Coenzyme Q10 was found to be efficacious in a RCT (Sándor et al., 2005) and 2 open label trials (Rozen et al., 2002, Shoeibi et al., 2017). Riboflavin (vitamin B2) is a precursor for flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) regulators of the mitochondrial electron transport chain. Two studies have shown high dose riboflavin to be an effective migraine prophylactic (Boehnke et al., 2004, Schoenen et al., 1998).

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Issues with Oral Prophylactic Migraine Treatments

Despite the advances in migraine prophylaxis in the past 30 years, there are still many challenges in successfully managing patient migraine outcomes. The side effects associated with the prophylactic drugs can be severe, putting patients off taking them for sustained periods of time. The adherence rate for prophylactic treatment regimen drops rapidly, from 41-95% at 2 months to 35-56% at 12 months, with adverse events cited as the most common reason for discontinuation (Hepp et al., 2014, Rahimtoola et al., 2003).

Additionally, there is the challenge of refractory patients that do not respond to at least 3 of 4 of the prophylactic treatment classes (beta-blockers, anticonvulsants, tricyclics, and calcium channel blockers). One study estimated the rate of refractory patients attending the headache unit at the University Clinic of Navarra at 5.1% (Irimia et al., 2011). However, there is considerable debate surrounding the clinical definition of refractory chronic migraine, there has been proposed definitions from the European headache federation (Martelletti et al., 2014) and the American headache society (Schulman et al., 2008), however, the ICHD-3 does not contain an official definition (IHS, 2018).

Injectable Prophylactic treatments for migraine

For patients that have failed three or more prophylactic treatment options, injections of onabotulinum toxin type A (BoNT/A) can be given by a headache specialist. The recommended treatment regimen is 31 bilateral intramuscular injections every 12 weeks (Diener et al., 2010). BoNT/A cleaves the SNAP-25 protein from the pre-synaptic cleft preventing vesicle, and thus neurotransmitter, release into the synapse (Dolly, 2003). Specifically, how and where BoNT/A interacts with migraine pathophysiology remains unclear, it has been suggested that it prevents the peripheral release of neuropeptides including GCRP and substance P from free nerve ending (Aoki, 2003), BoNT/A has been shown to have selective inhibition of mechanical (Burstein et al., 2014) and chemical stimuli (Zhang et al., 2016) on trigeminal C-fibre meningeal nociceptors (Melo-Carrillo et al., 2019). An alternative suggestion is that BoNT/A exerts central effects, by being retrogradely transported along the axon to inhibit the release of neurotransmitters at the cell body or spinal cord synapse (Antonucci et al., 2008, Hong et al., 2017).

Subcutaneous injections of local anaesthetic and steroids can be used to block the activity of the occipital nerves. This reduces activity in the TCC and is believed to reduce central sensitisation. Occipital nerve blocks have been shown to be a somewhat effective treatment for chronic migraine (Ashkenazi and Levin, 2007) although the data is inconsistent. Subcutaneous occipital nerve blocks are not included in the NICE recommendations for migraine treatment (NICE, 2019b) but have a favourable clinical outcome in tertiary care centres.

Recently there has been exciting developments in migraine prophylaxis with the CGRP monoclonal antibodies (mABs). These antibodies prevent GCRP receptor activation by either binding directly to the receptor or to free floating CGRP ligand in the extracellular matrix.

Ereumab is a fully human CGRP receptor monoclonal antibody. The phase III STRIVE clinical trial and the ARISE phase III trial showed dose dependent reductions of migraine days per month compared with placebo, (Goadsby et al., 2017, Dodick et al., 2018a), similar reductions have also been shown in real world audits (Lambru et al., 2020, Andreou et al., 2020). Erenumab has shown to have no serious side effects. Fremanezumab is a humanized GCRP peptide monoclonal antibody. Phase III clinical trials of episodic migraine patients Fremanezumab showed a reduction in migraine days (Dodick et al., 2018b). A similar reduction in monthly migraine days was also seen in chronic migraine patients (Silberstein et al., 2017). Galcanezumab is a humanized GCRP peptide monoclonal antibody. A phase III clinical REGAIN trial showed a dose dependent reduction migraine days compared with the placebo group (Detke et al., 2018). Eptinezumab is a humanized GCRP peptide monoclonal antibody. The PROMISE-1 phase 3 trial showed a dose dependent reduction of monthly migraine days compared with placebo (Saper et al., 2019).

Erenumab (Aimovig from Novartis), has been approved for use within the NHS in England and Wales for chronic and episodic migraine and chronic migraine for NHS Scotland (NICE, 2021). Fremanezumab (Ajovy from TEVA) has been approved for use within the NHS in England, Northern Ireland, Wales and Scotland for chronic migraine, and for episodic migraine in NHS Scotland (NICE, 2020b). Galcanezumab (Emgality from Eli Lilly) has been approved for use within the NHS in England, Wales and Northern

Ireland for chronic and episodic migraine (NICE, 2020c). The final CGRP mAB Eptinezumab (Vyepti from Lundbeck Seattle BioPharmaceuticals) is due to be submitted for approval in the UK.

Table 2: Summary of pharmaceutical treatments available for the treatment of migraine

	Medications	**Dose Suggestion**	**Mechanism**	**NICE recommendation**
Acute treatment	Triptans	Sumatriptan (50–100 mg)	5-$HT_{1B/D}$ agonists	Primary
	Simple Analgesia	Ibuprofen (400mg), Aspirin (900 mg), Paracetamol (1000 mg)	COX_{1+2} inhibitors	Primary
	Antiemetics	Metoclopramide (10 mg), prochlorperazine (10 mg)	D_2 receptor antagonist	Primary
	Ergot Alkaloids	N/A	5-HT_1 agonists	Not recommended
	Opiates	N/A	Opioid receptor agonist	Not recommended
Preventative treatment	Beta-blockers	Propranolol (80–160 mg daily)	B-adrenergic receptor antagonist	Primary
	Tricyclic antidepressant	Amitriptyline (25–75 mg)	serotonin, noradrenaline, dopamine transporter antagonist	Primary
	Anti-epileptics	Topiramate (50–100 mg daily)	$NA^{2+}{}_v$ antagonist GABA agonist	Primary
	BoNT/A	31x 5-unit injections	$SNAP_{25}$ Cleaving	Tertiary
	GCRP mAB	N/A	CGRP antagonists	Tertiary

Issues with Injectable Prophylactic Migraine Treatments

BoNT/A has proved to be effective and well tolerated in patients, with minimal side effects (Diener et al., 2010). However, the administration of BoNT/A is extensive and invasive, 31 intramuscular injections across the head and neck, makes the process unpleasant and potentially daunting prospect for the patient. Additionally, due to the location and neurotoxic potential these injections are required to be administered by a trained specialist at a tertiary headache centre, requiring a trip to the limited number of tertiary headache centres offering the service every 3 months.

Similar to BoNT/A, CGRP mABs are administered via subcutaneous injection. Although the injections are more frequent, every 4 weeks, it's only a single injection to the abdomen, thigh or upper arm, which can be self-administered by the patient, in the same fashion as insulin. The NICE evaluation of Erenumab highlighted the two major issues with the CGRP mAB uncertainty of long-term effectiveness and the prohibitive cost of the treatment (NICE, 2019a). The limited long-term efficacy and safety data is simply the result of CGRP mABs being a new treatment and will be investigated given time. Although the first long-term studies are starting to be published from the United States showing continued efficacy (Ashina et al., 2019). The NICE evaluation concludes that while erenumab was clinically effective, it had not shown to be more effective than BoNT/A, while the cost-effectiveness ratio was above the £20,000 to £30,000 (per quality-adjusted life years) considered cost-effective use of NHS resources (NICE, 2019a). As of February 2020 an appeal panel required further consideration of the cost-effectiveness of Erenumab specifically for BoNT/A non-responders (NICE, 2020a).

Chapter 1:
Introduction

Neuromodulation
The International Neuromodulation Society defines therapeutic neuromodulation as:

> *"The alteration of nerve activity through targeted delivery of a stimulus, such as electrical stimulation or chemical agents, to specific neurological sites in the body."(INS, 2013)*

Neuromodulation techniques can be invasive, requiring minimal surgery to implant a stimulating electrode directly onto a site of action or an external non-invasive device (Fig. 7) (Lambru and Lanteri-Minet, 2019).

Neuromodulation has advantages over traditional pharmaceutical therapies being targeted to specific regions of interests rather than system wide, as well as, having no drug-drug interactions, the result of both being usually much lower levels of side effects compared with traditional treatments. Neuromodulation also offers an additional different treatment option for refractory patients that have tried multiple preventative treatment options and found them to be ineffective (Lambru and Lanteri-Minet, 2019). Neuromodulation options for migraine can be invasive and non-invasive.

Invasive neuromodulation treatments for migraine
Occipital nerve
The greater occipital nerve arises from between the C1 and C2 vertebrae and provides innervation to the occipital scalp. The lesser occipital nerve rises from the C2 vertebrae and provides innervation to the skin of the lateral scalp posterior to the ear. The rational for its use as a treatment for migraine is the convergence of both trigeminal and occipital nerves on second order neurons in the TCC. By modulating the activity of the occipital nerve, the ascending second order neurons, receiving input from both trigeminal and occipital afferents can also be modulated. To stimulate the occipital nerve, usually two electrodes are implanted subcutaneously, at the back of the neck, across the branches of the greater and lesser occipital nerves. Supramaximal stimulation of the greater occipital nerve produces facilitation of second order neurons in the TCC (Bartsch and Goadsby, 2002). However, low frequency stimulation (3 Hz) of the greater occipital nerve did not modulate the trigeminal system (Jürgens et al., 2008).

Several large multicentre randomised control trials (RCTs) using implanted occipital nerve stimulation have been carried out. The ONSTIM RCT study compared occipital nerve stimulation (stimulation parameters: 0-10.5 V, 3-130 Hz, 60-450 μs) of three groups of patients: adjustable stimulation (0-10.5 V, 3-130 Hz, 60-450 μs, *n* = 33), a control group with a pre-set stimulation (1 minute per day stimulation, *n* = 17) and second control group that maintained medical management treatment (*n* = 17). After 3 months 39% of the adjustable stimulation had a 50% improvement in headache days vs 6% of the pre-set group and 0% of the medical management group (Saper et al., 2011).

A further study of 105 patients given occipital nerve stimulation for 12 weeks found no difference on the primary endpoint (>50% reduction in daily visual analogue scale) vs the sham group (*n* = 52), however, in patients seeing a 30% reduction there was a significant difference. Additionally, they showed significant improvements in; headache days, migraine days, pain relief and migraine related disability compared to sham group (Silberstein et al., 2012).

Two further single centre RCT have also been carried out on occipital nerve stimulation for chronic migraine that both found the treatment to be safe and effective (Serra and Marchioretto, 2012, Slotty et al., 2015).

High frequency Spinal cord stimulation

High frequency (>10 Khz) spinal cord stimulation (HF10 SCS) works by stimulating wide dynamic range neurones, in the dorsal horn of the spinal cord to inhibit nociceptive inputs (Cuellar et al., 2013). This stimulation protocol does not induce paraesthesia. HF10 SCS has been shown to be an effective neuromodulation technique in a number of chronic pain conditions including; cluster headaches and chronic back and leg pain (Lambru et al., 2016) (Kapural et al., 2015, Kapural et al., 2016, Van Buyten et al., 2013). Although a further RCT found its analgesic effect comparable to sham treatment (Perruchoud et al., 2013).

To treat primary headaches, HF10 SCS is implanted on the second and third cervical vertebrae to modulate V1 afferent nociceptive input at the trigeminocervical complex.

Three small studies have investigated FH10 SCS for chronic migraine. A retrospective study by Agostino *et al.* (2014) found 82% of the 17 patients implanted with the HF10 SCS had a 30% decrease in mean headache intensity. Improvements were also seen in mean migraine days (decreased from 28 to 9 days), quality of life (increased from 3.3 to 7.2) and number of patients not requiring analgesics (increased from 0.0% - 37.5%). Three patients suffered from infection, requiring the removal of the stimulator, and three patients had lead migration requiring correction (De Agostino et al., 2015).

Arcioni *et al.* (2015) implanted 17 refractory chronic migraine patients with HF10 SCS. Fourteen patients continued the treatment for 6 months, 7 patients had a 30% improvement in headache days (average reduction of 12.9 ± 5.3 days). Improvements were also seen in medication intake (from 64% overusing triptans to 36%), HIT6 score (decreased by 8.3), and headache intensity and frequency (decreased by 49% and 40% respectively. Two patients had lead migration and 1 had a lead fracture, 2 patients had infections and 3 reported discomfort from the implant (Arcioni et al., 2016).

Lambru *et al.* (2016) implanted HF10 SCS into 4 chronic migraine patients, as well as two SUNA patients and one patient with cluster headaches. Followed up at a mean 25 months, all 4 chronic migraine patients showed a 50% improvement in headache days, reverting to episodic migraine. One patient suffered a lead breakage resulting in worsening headaches, which was resolved following surgical replacement (Lambru et al., 2016).

Non-invasive

Supraorbital Nerve Stimulator

Transcutaneous electrical stimulators use electrical currents to directly stimulate the afferent supraorbital branch of the trigeminal nerve. Supraorbital nerve stimulators have been investigated as both an acute and preventative migraine treatment. As an acute treatment, 106 patients with acute migraine attacks were treated in an RCT (ACME). Overall, the change in pain intensity at 1 hour compared to baseline was significant in the treated group compared to sham treated (-59% vs -30% respectively). Reduction in pain intensity was significant in migraine without aura but not migraine with aura patients (Chou et al., 2019). In the randomised control trial of 67 patients with episodic migraine

(PREMICE) 20 minutes per diem supraorbital nerve stimulation for 3 months reduced headache days per month (6.94 to 4.88 days per month), as well as a reduction in acute medication intake. However, although the reduction within the treatment group was significant, when compared with the sham group the reduction did not reach significance (*P* = 0.054) (Schoenen et al., 2013).

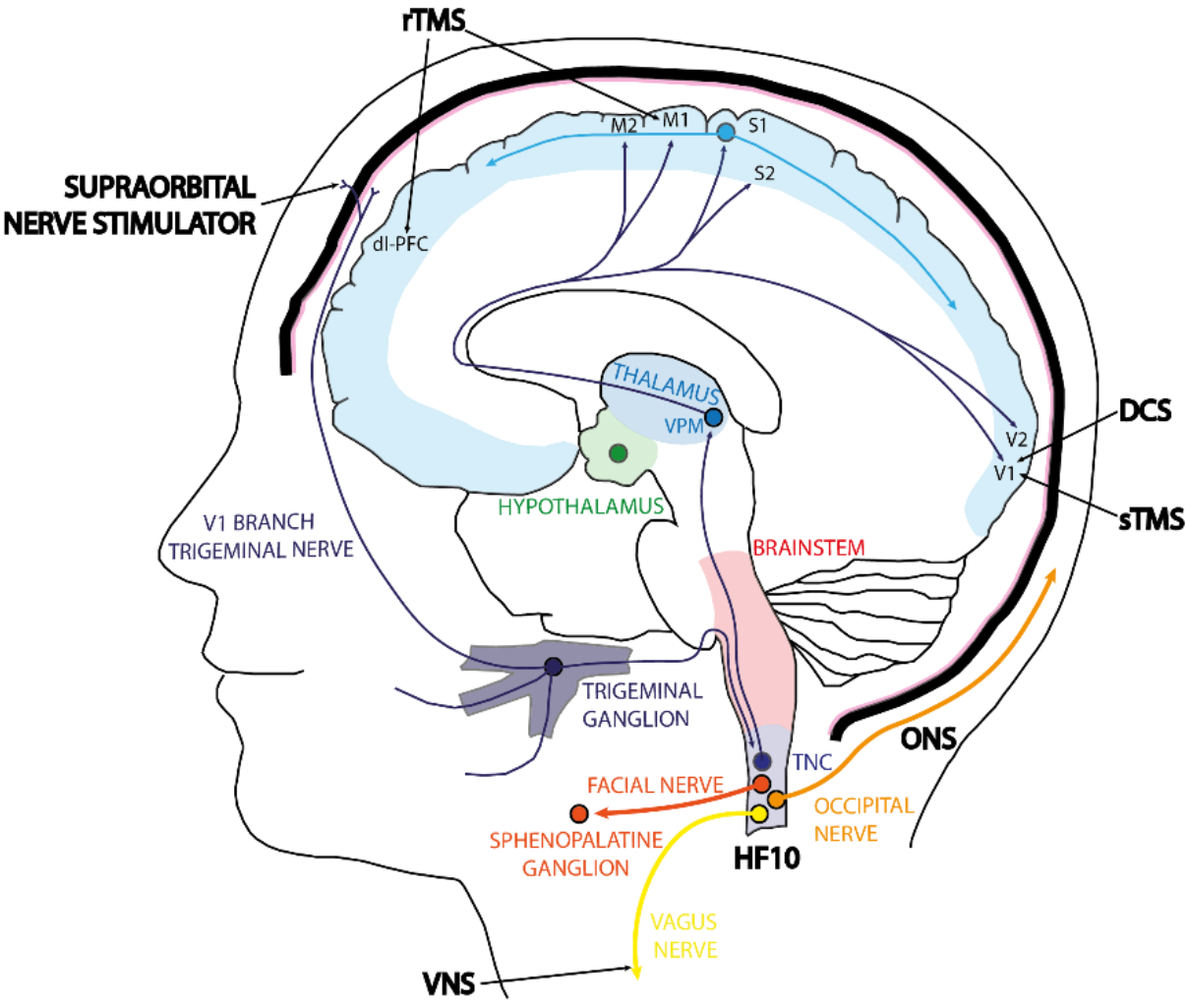

Figure 7: Sites of action of neuromodulation treatments for migraine*; invasive neuromodulation – occipital nerve stimulation (ONS), High frequency (>10 Khz) spinal cord stimulation (HF10 SCS), non-invasive neuromodulation – vagus nerve stimulation (VNS), supraorbital nerve stimulator, transcranial direct current stimulation (tDCS), repeated measures transcranial magnetic stimulation (rTMS) and single-pulse transcranial magnetic stimulation (sTMS).*

Transcutaneous Vagus nerve stimulator

The vagus nerve, the Xth cranial nerve, arises from the medulla, containing both afferent sensory (80%) and efferent motor (20%) fibres. The vagus nerve provides parasympathetic innervation to the autonomic nervous system, involved in a variety of autonomic functions including the respiratory, cardiovascular and nociceptive systems. The vagus nerve has been shown to be able to modulate trigeminal activity, inhibiting the response of TNC neurons to trigeminal stimulation in 48% of neurons, and facilitating activity in 29.5% of neurons (Lyubashina et al., 2012) and shown to be superior to sham at providing pain freedom up to 60 minutes post stimulation and therapeutic benefit up to 120 minutes (Tassorelli et al., 2018). Stimulation of the vagus nerve has also been shown to inhibit cortical spreading depression (Chen et al., 2016b) and substantially shorten visual aura (De Icco et al., 2019).

Commercially available devices are available that can stimulate the vagus nerve non-invasively by applying an electrical current transcutaneously to the neck. This treatment has been tested for the acute and preventative treatment of migraine. Acutely several studies have shown vagus nerve stimulation caused a 50% reduction of pain scores at 2 hours (in 22% (Goadsby et al., 2014), and 64.6% (Barbanti et al., 2015) of treated patients). As a preventative treatment, vagus nerve stimulation reduced headache days in chronic patients by 1.4 days (vs 0.2 days in sham treatment) (Silberstein et al., 2016) however, the PREMIUM RCT showed that nVNS treatment was not superior to sham stimulation as a preventative treatment for episodic migraine (reduction of 2.26 migraine days vs 1.8 days) (Diener et al., 2019). Real world experience has corroborated a lack of efficacy for non-invasive vagus nerve stimulation as an acute or preventative treatment for several chronic refractory primary headache conditions (Chronic migraine, chronic cluster headache and SUNA) (Trimboli et al., 2018).

Transcranial direct current stimulation

Transcranial direct current stimulation (tDCS) aims to non-invasively stimulate neurons in the cortex through the skull by applying weak electrical current (0.1-2 mA) to the scalp (Lambru and Lanteri-Minet, 2019). TDCS uses stimulation from a positive (Anode) and negative (Cathode) electrode placed over a target area to manipulate the membrane

potential of neurons in the cortex. Anodal stimulation depolarises the neurons causing cortical excitation, while cathodal stimulation hyperpolarises the neurons decreasing excitation in the cortex (Antal et al., 2004). TDCS has been shown to modulate activity in the motor and visual cortices in both animal and human models (Antal et al., 2004, Ward and Weiskrantz, 1969) and have prolonged effects (Bindman et al., 1964, Nitsche and Paulus, 2001).

Several studies have investigated tDCS as a prophylactic treatment for migraine. A pilot study treated 10 migraine without aura patients three times a week for 8 weeks, applying anodal tDCS to the visual cortex. A decrease in migraine attack frequency and duration (but not intensity) was seen following tDCS treatment compared to baseline, as well as a decrease in analgesic intake (Viganò et al., 2013). An RCT by Antal *et al.* (2011) applied cathodal stimulation to the visual cortex of 26 patients three times a week for 6 weeks. They showed no change in the frequency of attacks with tDCS treatment compared with the control group, duration of attacks was significantly reduced compared to baseline, however, not compared with controls, a significant reduction in intensity and number of migraine days was seen after treatment compared with the control group (Antal et al., 2011). A similar second RCT treated 10 migraine patients three times a week for 4 weeks, a significant reduction in the number and duration of attacks (but not the intensity) as well as the analgesic intake was seen when compared to baseline. However, when comparing with the control group no differences were seen in the number, duration or intensity of migraine attacks (Rocha et al., 2015). Several of the studies have shown significant improvements in therapeutic outcomes when compared to baseline, however as Rocha *et. al* (2015) shows, these results may not remain significant when compared against control groups. While tDCS has some evidence to suggest it may be an effective non-invasive treatment option for migraine, the current evidence remains mixed. Practically, it is a treatment that needs to be delivered in a clinical setting by experts and hence its use has not been widely adopted.

Chapter 1:
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TMS

Like transcranial direct current simulation transcranial magnetic stimulation (TMS) is a non-invasive neuromodulation technique that electrically stimulates the cortex of the brain. However, unlike direct current stimulation it stimulates the cortex without directly applying electrical cables on the scalp that could also stimulating the peripheral nociceptors, hence, preventing discomfort during application.

TMS uses an electromagnetic coil to produce a magnetic pulse that crosses the scalp and skull to produce an electrical current in the underlying cortex via electromagnetic induction. In 1820 Orsted showed that electrical current produced a magnetic force (Ørsted, 1820). In 1831 Michael Faraday showed the converse was true and a magnetic force was able to produce an electrical current in a process known as "electromagnetic induction". This relationship was formally described in 1873 by James Clark Maxwell in "A Treatise on Electricity and Magnetism" (Clark, 1873). TMS was first developed by Anthony Barker in Sheffield, initially by stimulating nerve trunks (Polson et al., 1982). The earliest devices capacitors could retain up to 200 V resulting in a:

> *"sensation and slight muscular contraction of the hand"* (Barker et al., 1979).

Further refinement of the technique has led to improvements in the device able to support magnetic outputs of 1.6 tesla (T) (Hess et al., 1987) (since improved to >2.5 T) and stimulate the motor cortex, producing movement of the arm or leg (Barker et al., 1985).

Early TMS devices could only produce single pulse TMS (sTMS) or pairs of TMS pulses (ppTMS) requiring time to recharge the capacitors. However, subsequent improvements lead to the development of repeated pulse TMS (rTMS). This protocol fires trains of hundreds or thousands of pulses across the scalp to produce long lasting effects in the activated neuronal tissue by neuronal plasticity. A newer form of rTMS, is theta-burst stimulation (TBS), was developed in 2005. TBS uses high frequency (50-100 Hz) bursts of pulses repeated at a lower frequency (5 Hz) to induce plasticity of the cortex (Huang et al., 2005).

TMS Mechanism of Action

Capacitors store an electrical current which is rapidly discharged through a conductive copper coil producing a magnetic pulse. If the coil is in close proximity to the electrically conductive neural tissue the magnetic pulse carries into the tissue, which acts as a secondary contact producing a field of electrical current, depolarising the local cortical neurons. The current induced by the magnetic pulse is perpendicular to the coil surface due to magnetic flux, at a maximum on the cortical surface and directly proportional to the current passed through the coil.

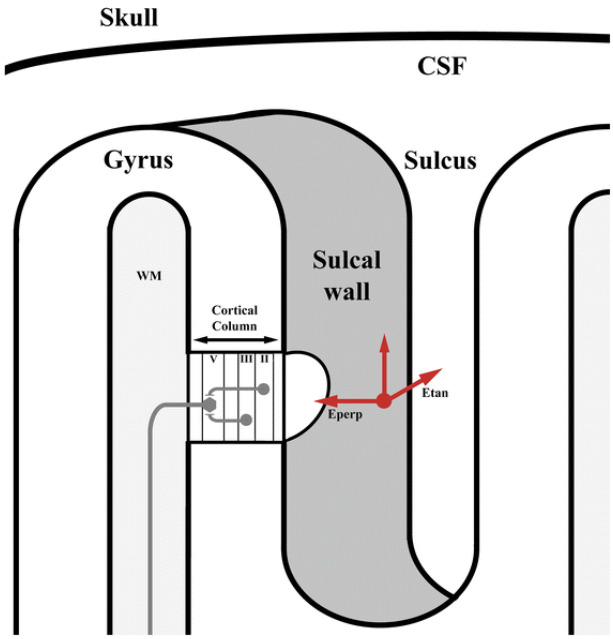

Figure 8: Orientation of Electrical field alters stimulation of cortical column in sulcal wall, *Included are neural elements (P2, P3, P5) that are possibly stimulated by the electric field component aligned with the axis of the cortical column. The electric fields perpendicular (Eperp) to the sulcal wall are more effective than electric fields orientated tangentially (Etan) (Janssen et al., 2015)*

Normal anatomical variation in the brains of healthy induvial has significant effects on the location and strength of sTMS' induced electrical current. While the TMS pulse passes unimpeded through the non-conductive skull, the thickness of the skull does affect the stimulation with an increased scalp-brain distance (as well as greater CSF thickness) resulting in weaker induced-current intensity (Lee et al., 2018). The resistive and capacitive electrical properties of the stimulated tissue can also affect alter the properties

of the induced current. A TMS pulse passing through multiple tissues showed increased field spreads and decreased electric field magnitudes in an *in-vivo* recording compared with *ex-vivo* recordings (Wagner et al., 2014).

The magnetic output required for the stimulation of pyramidal cells in the cortex causing a motor evoked potential (MEP) can be used as a threshold measure of cortical excitability (Koht and Sloan, 2018). Many studies report the stimulating parameters of the TMS pulse as a percentage of the MEP threshold rather than the tesla of the magnetic pulse or the calculated induced current. A similar threshold can be found in the visual cortex for the inducement of visual phosphenes, as well as auditory and somatosensory cortices. Stimulation of the motor cortex induces two volleys of activity, the first is activity resulting from direct stimulation of the pyramidal tract neurons (D-wave) and the second volley 1.5 ms later was indirect synaptic activation from other stimulated pyramidal neurones (I-wave) (Patton and Amassian, 1954, Kernell and Chien-Ping, 1967).

The magnetically induced electrical current rapidly dissipates, from the electromagnetic coil, with the greatest current intensity within 1-2cm of the coil. As an example, for a 9 cm circular coil, with a magnetic field of ~1 T, within ~5 cm of the coil surface the field strength halves, and within 10 cm is ~0.1 T (Epstein et al., 1990). This suggests that the direct stimulation of the TMS is highly localised to the surface of the cortex directly below the coil. However, the highly connected network nature of the cortex means that stimulation of one site has knock on effects intra- and inter-cortically.

The greatest stimulation produced by the electromagnetic coil is not at the centre of the coil (where there is an area of zero current), but around the circumference of the coil. Thus, a circular coil will -have a circular area of effect immediately below the coil, which has high penetration but makes fine focusing of the stimulation to a single precise location more difficult. This can be improved upon via coil shape and placement. A figure of 8 coil, has 2 circular coils side by side, this produces a larger peak of induced fields in the overlap between the two coils, allowing more precise stimulation (Fig. 9) (Thielscher and Kammer, 2004). In addition, by angling the coils this can allow for targeted stimulation of deeper brain structures. The orientation of the coil to the cortex also affects the induced electric current strength. The cortex is most sensitive to fields oriented perpendicular to

the surface and insensitive to parallel fields (Fig. 8) (Janssen et al., 2015, Laakso et al., 2013).

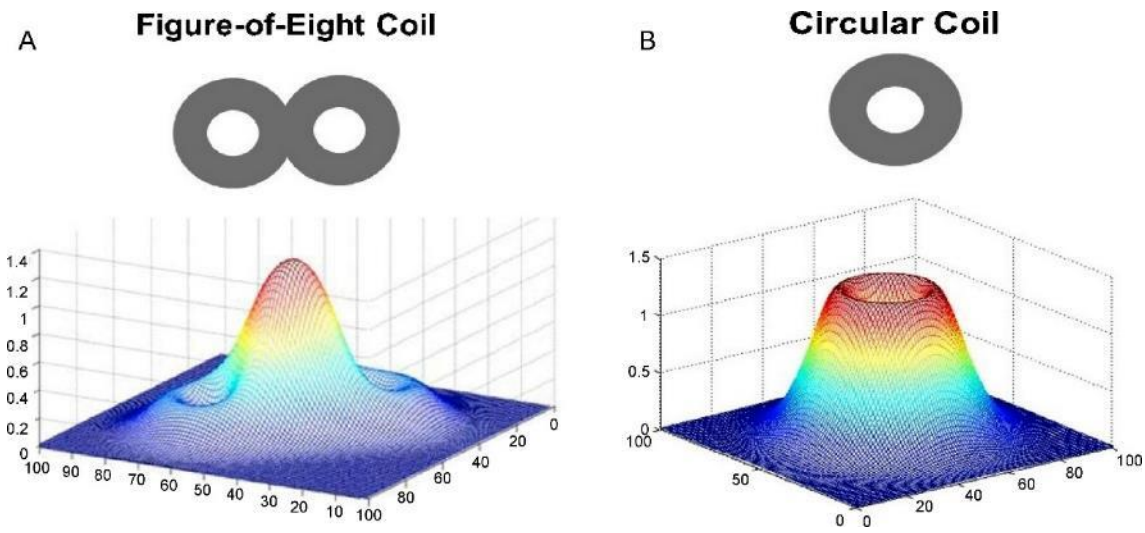

Figure 9: The strength of the electrical field induced below a circular (Right) and figure of 8-shaped coil (Left) (Giordano et al., 2012)

The risetime (the time taken for the magnetic pulse to reach peak amplitude) has an important effect on the energy required to reach threshold. Shortening the risetime, means that there is less charge required to produce an action potential, as less current leaks across the neuronal membrane. The duration of the magnetic pulse has a direct effect on the stimulation current applied to the cortex. The MEP threshold is decreased by 20% when the pulse is extended by 40% (Rothkegel et al., 2010). This has a practical consequence for the patient sensation, shorter (and higher amplitude) rTMS stimulations have been reported as being as more uncomfortable (Peterchev et al., 2017).

Several studies have investigated the physiological action of sTMS to understand its mechanism of action. When applied to the feline occipital cortex, paired pulse TMS has been shown to have differing modulatory actions depending on stimulus strength, below 50% of the MEP threshold, sTMS application caused a facilitation of neuronal activity for ~500ms followed by inhibition for several seconds. Stronger TMS stimuli (>50%) caused an initial inhibition of neuronal activation for ~100-200 ms, followed by rebound facilitation. (Moliadze et al., 2003). This result was later confirmed in a second study

showing facilitation of occipital neuronal activity following a 60-130% ppTMS stimulation, but inhibition at 15-50% stimulation (Moliadze et al., 2005). A similar finding of sTMS inducing transient facilitation followed by inhibition was suggested to be caused by direct stimulation of excitatory axons which excite inhibitory neurones leading to a rebound inhibition via metabotropic GABA receptors (Funke and Benali, 2010).

These findings are in accordance with paired pulse TMS effect on the motor cortex of humans, where stimulation with 2 pulses at the MEP threshold resulted in short interval intracortical facilitation. A subthreshold stimulation (70-80%) followed by a MEP threshold stimulation resulted in short interval intracortical inhibition. $GABA_A$ receptor agonist diazepam was found to increase inhibition and decrease facilitation. The mechanism of inhibition of the cortex was proposed to be due to a low-threshold $GABA_A$ receptor-dependent inhibitory pathway (Ilić et al., 2002).

The role of cortical GABA was further investigated by Murphy and colleagues (Murphy et al., 2016) in rodents, who found that sTMS (80-100%) activated inhibitory $GABA_B$ fibres in the upper cortical layers which in turn inhibited activity of dendritic pyramidal neurons in layer V. By blocking $GABA_B$ receptors they were able to prevent inhibitory effects of sTMS on the somatosensory cortex (Fig. 10).

Several studies have investigated the indirect effects of TMS on the thalamus. Application of 1Hz rTMS was shown to be able to increase activity in the mediodorsal nucleus of the thalamus (along with the hippocampus, putamen, pulvinar and insula) in depressed patients (Li et al., 2004). In a human study sTMS application to the frontal cortex was found to improve reporting of visual stimulus, which was suggested as evidence of reciprocal thalamocortical interaction (Amassian et al., 2008).

Cortical rTMS has been shown to effect activity of the hypothalamus, particularly as a treatment for depression. Application of rTMS to the prefrontal cortex, has been shown to modulate activity of the hypothalamus (George et al., 1999, Gur et al., 2004). Long term rTMS application was found to reduce glutamate and GABA levels in the hypothalamus, while increasing levels in the hippocampus and striatum in animal models (Yue et al., 2009).

Complex computational models have also been utilised to better understand how TMS interacts with single cells and larger cortical circuits. One large simulation modelled 33,000 neurons (with 5,000,000 synapses) arranged in a 3-laminae motor cortex and associated thalamic and reticular regions (Esser et al., 2005). They were able to replicate spontaneous neuronal activity, as well as sTMS evoked motor activity, previously been shown in animal models. In their model, they were able to replicate suprathreshold sTMS stimulation modulation of cortical activity. sTMS directly caused activity in laminae 2/3 and 5, this produced further activity in lamina 5 and 6 via intra and inter layer excitatory connections (AMPA and NMDA. In superficial layers 2/3, inhibitory interneurons (GABA) were activated preventing further activity. Subthreshold paired-pulse sTMS produced hyperpolarising of the synapses preventing firing in the superficial lamina, similar to what was previously found (Ilić et al., 2002). Given the similarity of findings to animal studies, these models may prove to be a highly useful alternative investigative tool in the future.

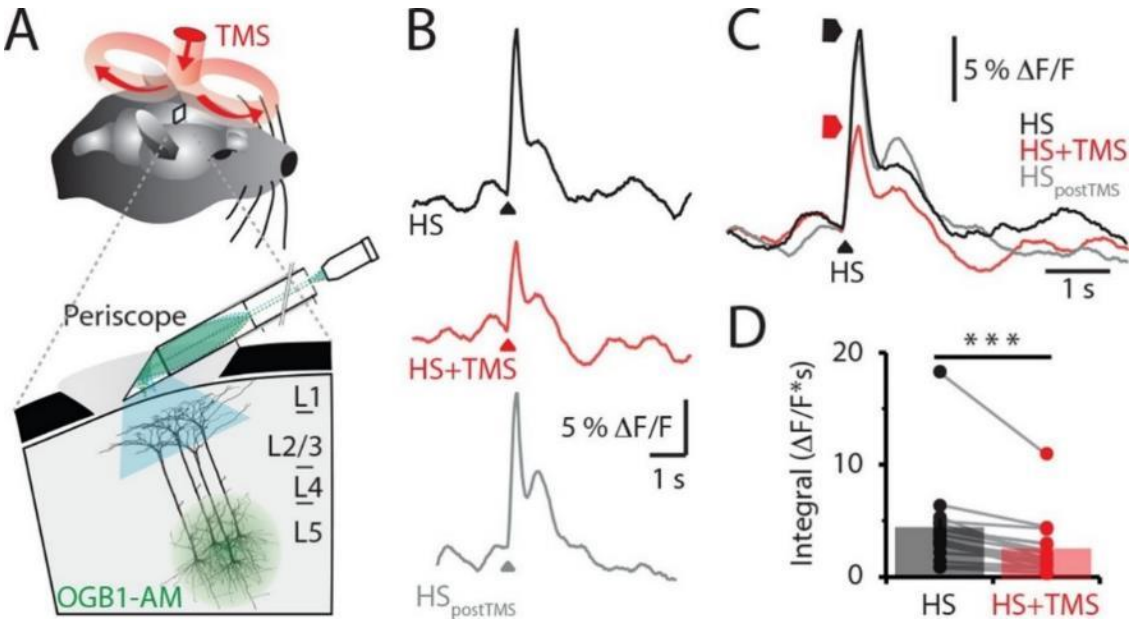

Figure 10: TMS inhibits sensory evoked Ca2+ activity in layer 5 dendrites. (A) Schematic of the experimental design*. Layer 5 pyramidal neurons were bulk loaded with OGB1-AM and dendritic Ca2+ activity was recorded using a flat-periscope configured horizontally and inserted underneath the TMS coil from the side. The TMS coil was placed above the dendrites in the hind paw region of the somatosensory cortex. (B) Typical dendritic Ca2+ response to hind paw stimulation (HP) alone (black) and during a single TMS pulse (red) and HP alone post-experiment (grey). (C) Overlay of traces in (b) and (D) graph illustrating the decrease in Ca2+ response during TMS (Murphy et al., 2016)*

Mechanisms of action of sTMS in Migraine

That sTMS is non-invasive, painless, effective and has minimal side effects suggests the great potential it has as a treatment for migraine, should it be more widely adopted. Although sTMS has been shown to be an effective and safe acute and prophylactic treatment for migraine, the precise mechanisms how it achieves the reduction in headache still remain to be fully elucidated.

STMS has also been shown to have modulatory effects on cortical and subcortical structures within the brain. Inhibiting both mechanically and chemically induced CSD's within the cortex and blocking both spontaneous and C-fibre activity of third order neurons in the VPM thalamic nucleus. However, sTMS has no modulating effect on the firing of second order trigeminocervical neurons. In addition, the inhibitory effect produced by sTMS could be blocked by pre-treating with the broad μ-opioid receptor antagonist, naloxone. This suggests that sTMS could interact with the endogenous opioidergic system to produce its anti-cephalalgic effect, similar to what has been shown in rTMS (Taylor et al., 2013, Taylor et al., 2012, de Andrade et al., 2011). Finally, the rise time of the magnetic pulse was an important factor in the response to sTMS. It was found that a rise of 170 μs was more beneficial than a rise of 100 μs, blocking more CSD's (Andreou et al., 2016).

TMS as an exploratory tool

In combination with other research techniques TMS can be a powerful research and diagnostic tool for studying cortical networks. When applied to the motor cortex (at or above threshold) TMS activates the corticospinal pathway, eliciting twitches in the muscle associated with the area of the cortex stimulated, which can recorded with electromyography (Barker et al., 1985). Applied to the occipital cortex, a similar strength stimulation will elicit a perceived flash of light known as a 'phosphene' in the retinal region associated with the area of the cortex stimulated. By adjusting the strength of applied stimuli, sTMS can be used to test the excitability of the cortex. The stimulation threshold required to evoke a motor evoked potential or for phosphene production can be used as a comparable measure of excitability (Valero-Cabré et al., 2017).

Combined with brain imaging techniques TMS has been used to investigate cortical connectivity and neuronal circuits in the healthy human brain. A TMS pulse can stimulate specific areas of the cortex and the functional connections mapped by recording the elicited response. To this end, TMS can be paired with a number of techniques including; electroencephalography (EEG), positron emission tomography (PET), single-photon emission computed tomography (SPECT), near infrared spectroscopy (NIRS), or Functional magnetic resonance imaging (fMRI), including blood oxygen level dependent (BOLD) signal (Wagner et al., 2007). As an example, cortical mapping using ppTMS has been used to investigate descending cortical modulation of the corticospinal pathway (Ferreri et al 2011).

TMS as an exploratory tool for migraine

Transcranial magnetic stimulation (TMS) MEP and phosphene thresholds have been used investigate cortical excitability in the motor and visual cortices respectively. Applied to the visual cortex several studies have shown a reduction of phosphene threshold in both migraine patients, both with and without aura (Aurora et al., 1998, Mulleners et al., 2001b, Gunaydin et al., 2006, Áfra et al., 1998, Gerwig et al., 2005, Chadaide et al., 2007, Aurora et al., 2003, Aurora et al., 1999b) suggesting cortical hyperexcitability. However, one contradictory study did find an increase in activation thresholds in the visual cortex, suggesting hypoexcitability (Bohotin et al., 2003). TMS thresholds have also been used to show that excitability may differ between the visual and motor cortices of the brain in migraine patients (Gunaydin et al., 2006, Bohotin et al., 2003, Werhahn et al., 2000, Áfra et al., 1998, de Noordhout et al., 1992, Bettucci et al., 1992). Therefore motor thresholds cannot be assumed to be a guide to visual cortex excitability (Stewart et al., 2001)

Magnetic suppression of perceptual accuracy (MSPA) test has been used as a technique for investigating excitability differences between migraine patients and controls. Since GABAergic inhibition of cortical neurons plays a role in the fine tuning of cortical processing, visual sharpness can be used as a test of the cortical GABAergic system. Letters are flashed to participants, after an interval (short: 40 ms, medium: 100 ms, or long: 190 ms) sTMS is applied to the occipital cortex, then participants asked to recall the flashed letter. Recall was impaired at when sTMS was applied at a median interval due

to the activation of inhibitory pathways. However, there was no impairment in recall when sTMS was applied after a long or short interval (Mulleners et al., 2001a), (Aurora et al., 2007, Chronicle et al., 2006).

A similar technique, metacontrast visual masking, can also be used to investigate inhibitory actions in the visual cortex. Using metacontrast it was found that migraine with aura patients were less susceptible to visual contrast than migraine without aura patients or controls, suggesting impaired cortical inhibition (Palmer et al., 2000). In the motor cortex it was found that the cortical silent period elicited by TMS was reduced in migraine with aura patients than healthy controls, leading to the conclusion that there is reduced cortical inhibition leading to increased excitability (Aurora et al., 1999a).

Repeated Measures TMS

As well as being delivered single pulses, TMS can be delivered in trains of hundreds or thousands of pulses to produce long lasting modulation in the neuronal tissue. The modulatory effect of the pulse trains can be variable depending on the frequency of pulses applied, low frequency (LF) firing (<1 Hz) has been shown to inhibit cortical activity while high frequency (HF) firing (>1 Hz) produced facilitation of cortical activity. However, in migraine with aura patients, a paradoxical facilitation of cortical excitability was seen at 1Hz (low-frequency) rTMS stimulation to the occipital (Brighina et al., 2002), extra-striate (Fierro et al., 2003) and motor cortices (Brighina et al., 2005), which was not seen in normal subjects. This facilitation supports the hyperexcitability model of migraine. Brighina *et al.* (2005) suggested this may indicate a failure of GABAergic inhibition, however it could additionally be due to lower stimulation thresholds for glutamatergic neurons (Brighina et al., 2005).

RTMS has been shown to be effective in several disorders, with definite efficacy shown for pain (HF rTMS applied to the contralateral motor cortex), depression (HF rTMS applied to the dorsolateral prefrontal cortex), and post-stroke recovery (LF rTMS to the contralateral motor cortex). Probable efficacy has also been shown for improving quality of life and pain in fibromyalgia (HF rTMS of the motor and prefrontal cortex respectively), improving motor function in Parkinson's disease (HF rTMS bilaterally to the motor cortex), post-traumatic stress disorder (HF rTMS to the dorsolateral prefrontal

cortex) and non-fluent aphasia post-stroke (LF rTMS to the inferior frontal gyrus) (Lefaucheur et al., 2020). The analgesic effect of rTMS applied to the motor cortex or dorsolateral prefrontal cortex can be blocked via the application of naloxone (Taylor et al., 2013, Taylor et al., 2012, de Andrade et al., 2011). This suggests that top-down endogenous opioids may play a role in rTMS' analgesic effect.

TMS has been shown to affect pain thresholds in animal models and humans. High frequency rTMS, applied over a large cortical area induced allodynia in rodent models (Ambriz-Tututi et al., 2012), however, when applied to the motor cortex of patients with chronic neuropathic pain it resulted in a reduction of acute pain (Lefaucheur et al., 2010). Low intensity rTMS has also been shown to improve pain thresholds in fibromyalgia patients (Maestú et al., 2013). The site of application appears to plays an important role, as paired pulse TMS applied to the secondary somatosensory cortex or the occipital cortex had no effect on human pain perception, but there was a facilitatory effect when applied to the somatosensory cortex and an inhibitory effect when applied to the medial frountal cortex (Kanda et al., 2003).

Theta-burst TMS

TBS uses high frequency (50-100 Hz) bursts of pulses repeated at a lower frequency (5 Hz) to induce plasticity of the cortex (Huang et al., 2005). The induced plasticity is similar or greater than induced by rTMS (Nyffeler et al., 2006). The stimulation protocol mirrors the 5 Hz theta stimulation pattern which is used to induce long-term potentiation in hippocampal brain slices (Larson et al., 1986).

Two protocols for TBS have been developed; continuous TBS (cTBS) applies bursts of pulses in an uninterrupted train, producing a long-term depression (LTD)-like depression in cortical excitability (Di Lazzaro et al., 2005). Intermittent TBS (iTBS) uses a period of bursts of pulses with a resting interval interspersed. This protocol produces a long-term potentiation (LTP)-like facilitation of cortical activity. As with LTD and LTP, the depression and facilitation of the motor cortex produced by TBS is dependent on NMDA receptor activity (Huang et al., 2007). In the visual cortex cTBS increased the threshold for producing visual phosphenes, consistent with a depression of cortical excitability, however iTBS had no effect on phosphene threshold (Franca et al., 2006).

rTMS for migraine

Several studies have been carried out to investigate rTMS as a prophylactic treatment for migraine. A case study of two patients in an RCT study for the treatment of major depressive disorder with rTMS reported an improvement in daily headaches. The patients had a prior diagnosis of daily headaches, unrelated to the major depression for which they were enrolled in the trial. Given 5 treatments of rTMS (10 Hz, 3000 pulses per session, 120%) per week for 4-6 weeks to the left dorsolateral prefrontal cortex. They saw a reduction in migraine attack severity and frequency as well as improvement in depression scores, which persisted up to a moth (O'Reardon et al., 2007)

In a pilot study of 11 chronic migraine patients, rTMS (20Hz, 10x 2 second trains, 90%) was applied to the prefrontal cortex in 12 sessions. rTMS reduced the number and severity of headache attacks as well as the amount of abortive medications, which was not seen in the placebo group. Speculated may be due to modulation of serotonergic systems, or the cortico-limbic connections (Brighina et al., 2004).

Armin *et al.* (2020) also investigated high frequency rTMS applied to the left dorsolateral prefrontal cortex in an open label study. Fourteen migraine patients (12 migraine without aura, 2 migraine with aura) were given 5 sessions of 900 rTMS pulses (5Hz, 1x 3-minute train, 100%) over 1 week and compared against 19 control patients (17 migraine with aura, 2 migraine without aura) given a sham treatment (5Hz, 1x 3-minute train, 50%). Improvements were seen in the attack frequency (3.1 reduction vs 1.5), the number of migraine days (3.8 days vs 2.8 days) and the impact of headache (15.3 reduction vs 6.1) (Amin et al., 2020). Concluding that rTMS is an attractive, well tolerated option for migraine prophylaxis.

Misra *et al.* also found positive results using high frequency rTMS to the left dorsolateral frontal cortex. 51 patients (50 migraine without aura, 1 migraine with aura) who had at least 7 attacks per month, were given three sessions of 600 rTMS pulses (8 Hz, 10x 2 second trains, 70%). Significant improvements in attack frequency and severity were seen, as well as a reduction in the number of acute medications. A visual analogue scale was used to asses patient headache, 50 patients (98%) reported a 50% improvement in the first and second weeks (Misra et al., 2012).

A further RCT study by the same group applied three sessions of 600 rTMS pulses (8 Hz, 10x 2 second trains, 70%) to the motor cortex (hotspot for the right digiti minimi) in 100 patients (93 migraine without aura, 7 migraine with aura). Both rTMS and sham stimulation groups showed a reduction in headache frequency compared to baseline reaching the nadir in the first week, the rTMS group remained reduced until the 5th week but the sham stopped being significant after the 3rd week. Additionally, the severity and frequency of attacks as well as the functional disability and the analgesic consumption were improved in the rTMS group compared to the sham treatment group (Misra et al., 2013).

However, several further RCT have contradicted these studies, finding little effectiveness as a treatment for migraine. A smaller RCT applied 1600 high frequency rTMS pulses (10Hz, 110%, 32x 5 second trains) to the dorsolateral prefrontal cortex 14 chronic migraine patients in 23 sessions over 8 weeks. No significant improvement was seen in the rTMS treated group however a significant improvement (58.1%) in headache days was seen in the sham treated group (Conforto et al., 2014).

Teepker *et al.* (2010) investigated low frequency rTMS for the treatment of migraine. Applying 1000 pulses (1Hz, 2x 500 pulse trains, 100%) over the vertex of 27 patients (14 migraine without aura, 13 migraine with aura) over 5 consecutive days. A reduction in headache days and migraine days was seen in the treatment group compared to baseline, however this was not significant when compared to the sham treatment. No significant change was seen in either the intensity of the headache attacks or the amount of analgesics taken. They concluded that rTMS treatment well tolerated but not effective for prevention of migraine (Teepker et al., 2010).

Of the 5 RCT discussed here (Table 3), they each used very different stimulation protocols, making direct comparison difficult. The site, intensity, and parameters of the applied rTMS pulses differed significantly in each of the RCT's, any of which could be the deciding factor in the effectiveness of the treatment. The only consistent feature separating the successful and unsuccessful RCT's appears to be the intensity of the stimulus. The successful treatments all used a stimulation intensity below the motor threshold, whereas the 2 unsuccessful RCT's both used stimulus intensity at, or above the motor threshold. Repeated measured TMS may yet be proven to be an effective treatment

for migraine, however, optimisation is still required to find the optimal stimulation protocol and placement.

Table 3: Summary of RCT's into rTMS as a treatment for migraine

RCT Study	Brain Area	№ sessions	Freq	Trains	Strength	Outcome
Armin *et al.* 2020	L dl PFC	5 (1 week)	5 Hz	1x 3 mins	100%	Effective
Misra *et al.* 2012	L dl PFC	3 (1 week)	8 Hz	10x 2 mins	70%	Effective
Misra *et al.* 2013	M1	3 (1 week)	8 Hz	10x 2 mins	70%	Effective
Conforto *et al*. 2014	L dl PFC	23 (8 weeks)	10 Hz	32x 5 sec	110%	Ineffective
Teepker *et al*. 2010	Vertex	5 (1 week)	1 Hz	2x 500 sec	100%	Ineffective

TBS for migraine

At the time of writing, only two small studies have been published into the efficacy of TBS as a prophylactic treatment for migraine. The first by Chen *et al.* (2016) investigated continuous theta-burst stimulation in 9 migraine patients. cTBS, applied to the primary motor cortex was applied in 20 sessions over a 4 weeks period (3x 50 Hz pulses at 80% intensity, for 40 seconds with 200 ms intervals) and patients monitored for a further 4 weeks following treatment. A reduction was seen in both headache (13.4 ± 10.1 days to 8.7 ± 10.1 days) and migraine days (8.6 ± 8.7 days to 1.0 ± 1.6 days) during and following treatment compared to baseline (Chen et al., 2016a).

Sahu *et al.* (2019) applied intermittent TBS to the Dorsolateral prefrountal cortex (DLPFC) of 20 migraine patients, comparing to 21 patients receiving a sham treatment. iTBS session were given *bis in die* (20 trains of 600 pulses at 80% intensity with 3x 50 Hz pulse bursts and 8 seconds intervals) for a total of 10 sessions over 5 consecutive days, patients were monitored for 12 weeks following treatment. A reduction in frequency, duration and severity of migraine headaches was seen in the iTBS group compared to the sham group with the greatest reduction seen in the second week immediately following treatment.

Both studies suggest that TBS may be a safe and effective prophylactic treatment for migraine, however larger scale studies would be required to confirm these findings.

Single pulse TMS

Instead of using trains of hundreds or thousands of magnetic pulses, single-pulse TMS (sTMS) uses single or pairs of pulses to achieve an effect. This has the advantage that the coils do not have the same issues with heating as the rTMS devices and therefore don't require the large cooling units. This means sTMS device can be smaller and more lightweight (Fig. 11), making it portable and suitable for home-use, a revolution in the development of TMS treatment.

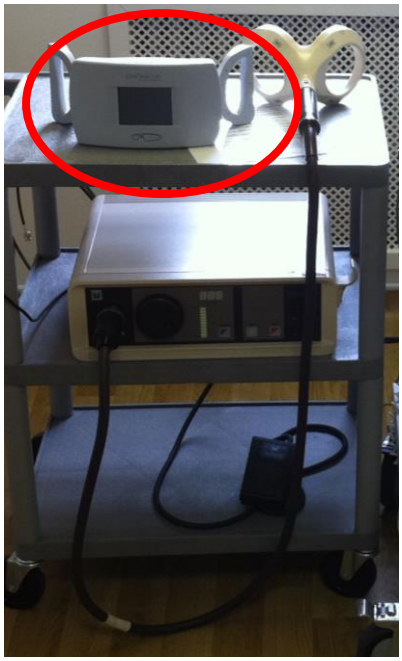

Figure 11: Side by side comparison of diagnostic rTMS and portable sTMS (circled in red) (Shields, 2012)

Clinical uses of sTMS

In addition to migraine sTMS has been tested for its efficacy as a treatment for several further neurological conditions. sTMS has also been targeted at the motor cortex in an effort to interrupt pain processing, however no significant differences in pain processing

of noxious heat pain were recorded when sTMS was applied pre or post stimulus (Kisler et al., 2018). sTMS has been shown to have positive clinical outcomes as an add-on therapy for patients with severe depression (Conca et al., 2000). In patients with Parkinson's disease, sTMS (to the motor cortex) was shown to reduce the reaction time to a visual signal (Pascual-Leone et al., 1994) and reset tremor when applied to the Motor and supplementary motor areas (Lu et al., 2015).

Clinical studies of sTMS for Migraine

In 2010 Lipton *et al.* carried out a randomised, double-blind, parallel-group, two-phase, sham-controlled, multi-centre study. Eighty-two migraine with aura patients were treated for at least one migraine attack with sTMS and were compared against a further 82 migraine with aura patients treated with a sham stimulation. Pain freedom 2 hours after treatment showed a 17% therapeutic gain in the sTMS vs the sham treatment. This appeared to be a sustained effect with greater proportion of the sTMS group remaining pain free 24- and 48-hours post treatment (Fig. 12). There were no reported serious adverse events, while minor side effects included headache, migraine, sinusitis and paraesthesia, and were not significantly different between the two treatment groups (Lipton et al., 2010).

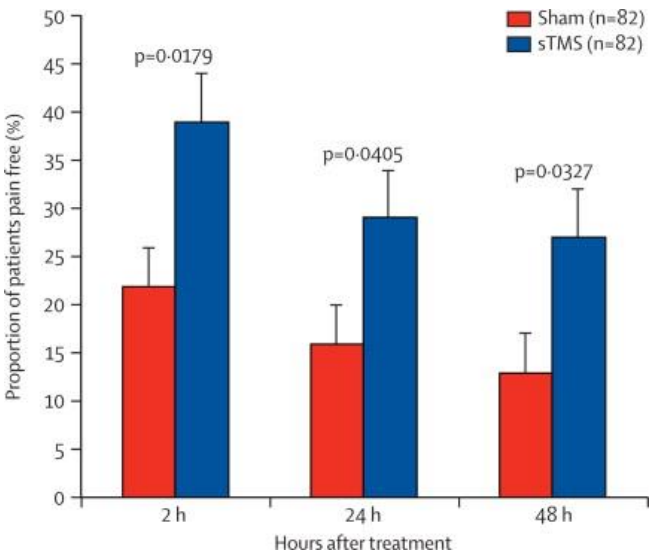

***Figure 12:** Pain-free response at 2 h, 24 h, and 48 h on active and sham treatment Error bars = SE (Lipton et al., 2010)*

In addition to being investigated as an acute treatment, several post-marketing studies have additionally looked at sTMS as a prophylactic treatment for migraine. Data from a 3-month UK post market pilot program showed pain relief in 62% of 190 patients (both migraine with and without aura). Headache days per month were improved in both episodic and chronic migraine patients. As well as, reduced attack duration, sTMS showed efficacy in relieving associated migraine symptoms, and a reduction in disability (Bhola et al., 2015).

Following this, the eNeura SpringTMS Post-Market Observational U.S. Study of Migraine (ESPOUSE) also investigated the efficacy of sTMS as a preventative treatment for migraine. A multi-centre, prospective, open label, observational study involved 217 patients who used the sTMS device daily for 3 months. Overall, a significant reduction in headache days was seen (-2.75 ± 0.40 from a baseline of 9.06 days) (Fig. 13), and 46% of patients achieved at least a 50% response rate. There was also a reduction in acute medication use and HIT-6 disability scores (Starling et al., 2018).

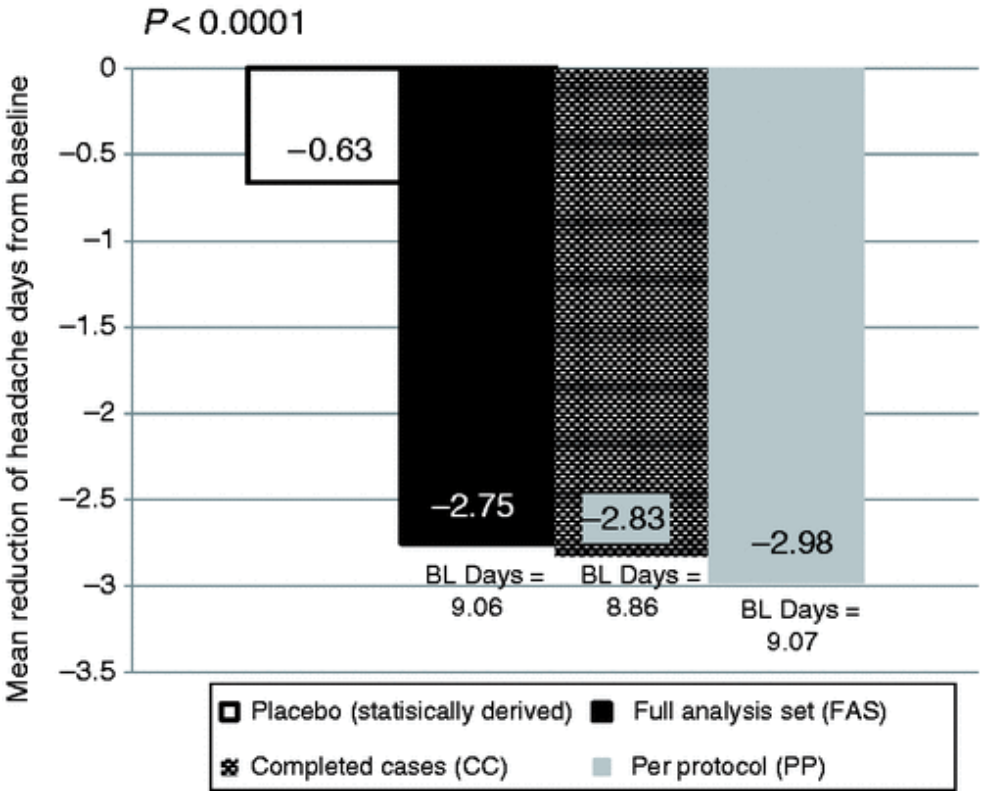

Figure 13: Primary effectiveness endpoint: Mean reduction in headache days(Starling et al., 2018)

sTMS' efficacy as a preventative treatment has also been studied specifically in adolescents (12-17 years old), in an open label pilot. Twelve patients completed the trial, with a further 9 failing to complete the study (most common reason for not completing was not returning or completing the baseline headache diary). Patients gave themselves 4x 0.9T pulses twice a day (2 pairs of 2 pulses separated by 15 minutes) for 12 weeks, with further pulses available for acute treatment. Use of the device was reported as feasible and tolerable, however the gap between treatments proved challenging and was suggested to be minimised. Furthermore, there was a significant reduction in headache days per month (-4.5 ± 1.7 days) and MIDAS score (-36 ± 14) (Irwin et al., 2018).

A smaller study from Canada tested paired pulse sTMS on 42 migraine patients (both with and without aura) as soon after the start of an attack as possible. After 1 round of 2 paired pulses of either high stimulation (50% of the maximum output) or low stimulation (30% maximum output) (using a Cadwell stimulator, max output; 2.3 T, 187 V) 69% of patients showed improvement, 87% improvement after 2 rounds and 82% after 3 rounds, suggesting a cumulative effect of TMS. They also suggested a larger effect in migraine with aura patients as 100% (10/42) reported immediate pain relief. Finally, they suggested an autonomic component, with heart rates dropping from 79.05±10.27 to 72.89±11.35 beats/min (Clarke et al., 2006). However, this study was not well controlled, the high vs low stimulation group were not shown to be significantly different and there was no control group not receiving any TMS treatment, thus the reported pain freedom 24 hours post treatment could just as easily be spontaneous recovery. There are also additional factors that would affect the outcomes that are not clearly reported including; episodic vs chronic migraine sufferers, additional acute and preventative medications taken, with aura vs without aura. It also involves a small number of patients and potentially lacks power.

As with the acute treatment there were no reported serious adverse events. The common adverse events reported included; light-headedness (3.7%), tingling (3.2%), and tinnitus (3.2%).

Government approval of sTMS for Migraine

As of 2014 the National Institute for Health and Care Excellence (NICE) and the U.S. Food and Drug Administration (FDA) approved the use sTMS for the treatment and

prevention of migraine (NICE, 2014b, Heetderks and Pena, 2016). As of 2019 the FDA has extended the use of sTMS to include the treatment of children over 12 years old (Marler and Pena, 2019).

sTMS Safety

No major side effects to sTMS have been reported with by patients undergoing sTMS treatment. With no major or unexpected side effects reported by any of the literature. Some of the minor side-effects include; tingling, light-headedness, drowsiness, tiredness, mild dizziness. A recent study found that single pulse TMS applied to Broca's area and the primary motor cortex induced scalp pain. This was believed to be stimulation of the peripheral nociceptors rather than top-down modulation. The stimulus required to produce scalp pain was significantly lower than the motor threshold (Tani et al., 2020).

A comparison of the incidence rate of mild adverse events was carried out between the different forms of TMS, including rTMS, TBS and sTMS, finding mild adverse events that were less likely to occur after sTMS (Dodick et al., 2010).

In 2010 Dodick *et al.* reviewed 2 decades worth of published literature on the reported adverse effects of sTMS and reported that:

> *"Tens of thousands of subjects have undergone TMS for diagnostic, investigative, and therapeutic intervention trial purposes with minimal adverse events or side effects. No discernible evidence exists to suggest that sTMS causes harm to humans. No changes in neurophysiological function have been reported with sTMS use."*

Concluding that sTMS is a safe non-pharmaceutical, non-behavioural treatment option for migraine patients (Dodick et al., 2010).

sTMS costs

Calculated costs to the NHS of one year of treatment with sTMS was compared to the conventional prophylactic treatment, BoNT/A. When the treatment was effective the costs for an individual funding request in the first year were £2923 for BoNT/A and £1466

for sTMS. sTMS has the potential for significant cost saving for the treatment of chronic refractory migraine patients (Brüggenjürgen et al., 2016).

Chapter 2: Acute direct effects of sTMS application on Cortical Neuronal Activity

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Acute direct effects of sTMS application on Cortical Neuronal Activity

Introduction

The cerebral cortex plays a particularly important role in the pathophysiology of migraine. As well as the processing of afferent nociceptive signals from the trigeminal nerve in the somatosensory cortex, migraine aura is believed to be a purely cortical event. Although migraine was previously believed to be a purely vascular disorder (Wolff, 1948), it is now considered to be primarily a dysfunction of the CNS with aberrant peripheral input (Parsons and Strijbos, 2003).

The cerebral cortex (from the Latin for 'bark') is the superficial layer of the brain comprising ~40% of the brain's mass, containing 10^{11} neurons (1 trillion) and 10^{15} connections (10 quadrillion). The human cortex is approximately 2-3 cm thick, however, thickness varies by location, and is subject to neuroplastic changes (Fischl and Dale, 2000). The precise structural and functional organisation of neurons in the cerebral cortex is exceptionally complex and beyond the scope of this thesis, however, a brief overview is necessary to understand the interactions with sTMS.

The cortex is comprised of repeating units called cerebral columns, a set of interconnected vertical chains of neurons that respond to nearly identical receptive fields, i.e. responding to the same patch of skin, area of the retina, auditory frequency etc (de Lorente, 1933). Although there are differences between areas of the cortex, there is a broadly uniform structure, allowing for rapid expansion during development and some limited remapping of inputs to new areas of cortex as necessary. Each column is comprised of six laminae, from I at the surface to laminae VI just above the white matter (Mountcastle, 1998).

The cortex broadly contains two types of neurons; the excitatory pyramidal neurons and the inhibitory stellate neurons. Small and medium pyramidal neurons form subnetworks within and between layer of the cortex. Pyramidal neurons responding to the same receptive fields, connect to one another, allowing activity of similarly firing neurons to be enhanced. Large pyramidal cells form longer connections between areas of the cortex or to other sub-cortical structures. Cortical activity is controlled with Gamma Aminobutyric Acid (GABA)ergic interneurons, found in laminae II-IV (Fröhlich, 2016), which form short range inhibitory connections within a layer of the cortex

indiscriminately to all neurons. This means activity of neurons outside of an activated sub-network are dampened.

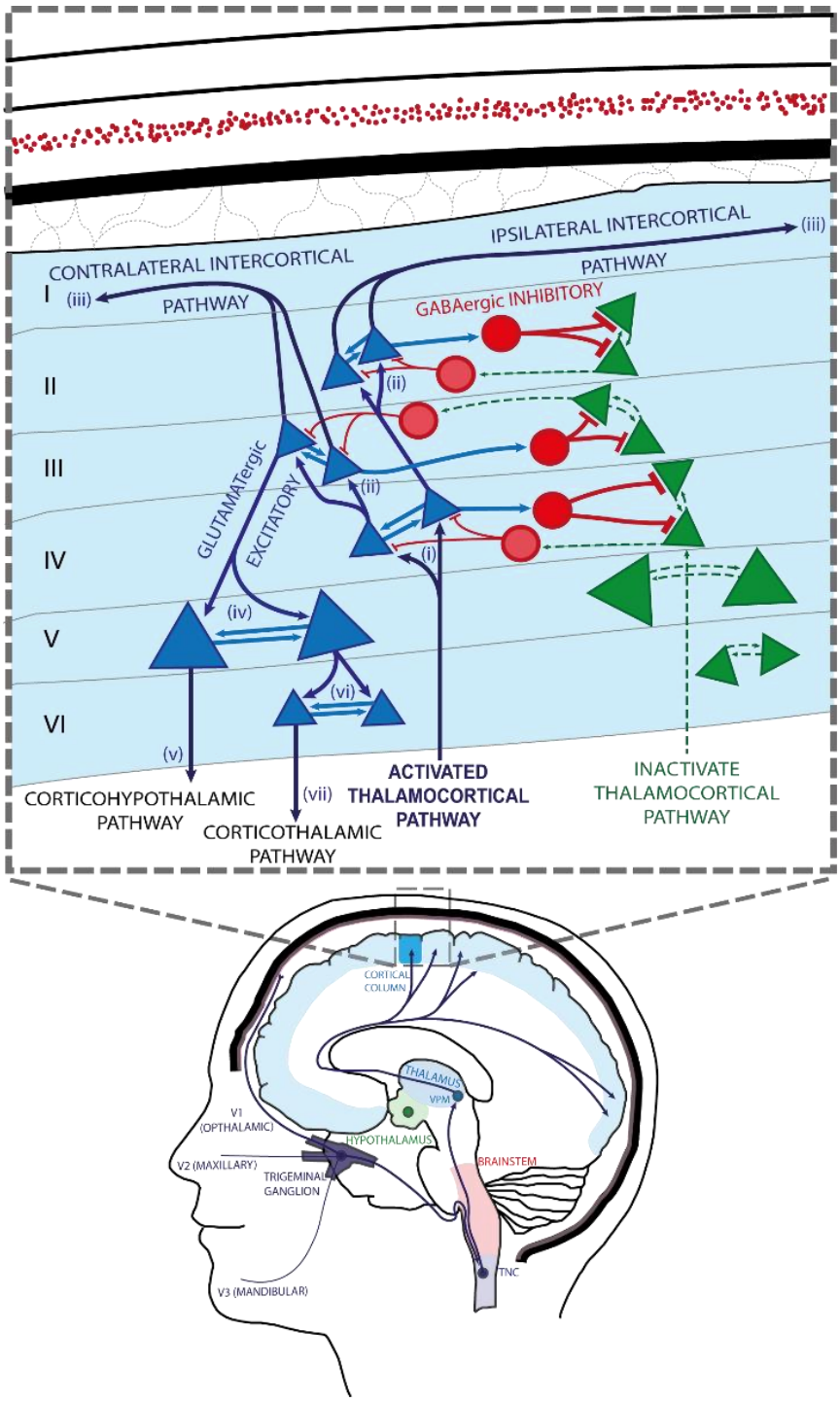

Figure 14: Summary of lamina structure of generic cerebral cortex column. *Activated thalamocortical pathway from the ventroposteromedial (VPM) thalamus activates the cerebral column (blue), while inhibiting neighbouring inactive cerebral column (green) through GABAergic inhibitory interneurons (red) in laminae II – IV. Activated cerebral column has projections intercortically from laminae II and III through lamina I and subcortically from laminae V and VI. Adapted from (Shipp 2007).*

The majority of third order afferent fibres from the thalamus project to the internal granular layer (lamina IV) of the cortex (Fig. 14i), with a small majority connecting to the multiform layer (lamina VI). The subnetwork of pyramidal cells in lamina IV (that receive input from 3rd order thalamic cells) have connections upwards to the external granular and external pyramidal layers (lamina II and III respectively, Fig. 14ii) that are often considered together, as both project intercortically via transverse pyramidal fibres in the plexiform layer (lamina I, Fig. 14iii). Pyramidal cells in lamina II project to the ipsilateral cortex while pyramidal cells in lamina III, project to the contralateral cortex, as well as to the internal pyramidal layer (lamina V, Fig. 14iv). The internal pyramidal layer projects to the basal ganglia and other sub-cortical regions (Fig. 14v) as well as to the multiform layer (lamina VI, Fig. 14 vi). The multiform layer is the deepest layer of the cortex directly above the white matter. The multiform layer projects back to the same area of the thalamus as the original input, thus completing the cortico-thalamic feedback loop (Fig. 14vii) (Shipp, 2007).

Cortical Processing of Pain

The "pain matrix", comprised of the primary and secondary somatosensory (S1/S2) cortices and the insula cortex, processes the afferent trigeminal nociceptive signals from the VPM thalamic nucleus and perceiving the headache pain. Additional cortices also receive afferent input from the posterior and lateral posterior/dorsal thalamic nuclei and are believed to be involved in giving rise to the associated symptoms of migraine. These include the visual, motor, parietal association, retrosplenial, auditory and olfactory cortex, which have been suggested to play a role in the sensory sensitivities, cognitive performance, and motor associated migraine symptoms (Noseda et al., 2011).

Repeated migraine attacks may cause neuroplastic structural changes in the cortex. Diffusion tensor imaging (DTI) was used to study the somatosensory cortex in migraine. Researchers found a cortical thickening in migraine patients when compared to age and sex matched controls, particularly in the caudal somatosensory cortex where the trigeminal area is processed (DaSilva et al., 2007). Further studies have suggested that cortical areas in which thickening was observed, also showed stronger functional activation that correlated to the frequency of headache attacks (Maleki et al., 2012a).

Cortical spreading depression and the role of cortical activation in migraine

The role of the cortex in migraine has been mainly researched due to cortical spreading depression (CSD), which is believed to be the underlying biological substrate of migraine aura. In 1941 Lashley mapped his own visual aura suggesting a disruption of function of the visual cortex (Lashley, 1941). In 1944 Leão reported on waves of spreading depolarisation in the cortex of rabbits (Leao, 1944), while the following year Leão and Morrison suggested this may be the cause of migraine aura saying;

> *"Much has been written about the vascular phenomena both in clinical epilepsy and the presumably related condition of migraine. The latter disease with the marked dilation of major blood vessels and the slow march of scotomata in the visual or somatic sensory sphere is suggestively similar to the experimental phenomenon here described."* (Leao and Morison, 1945).

In 1959 Milner wrote a short note further linking together Lashley's aura symptoms with Leão's CSD (Milner, 1958). It is now widely accepted that CSD is the physiological correlate of migraine aura. The characteristic alteration's in cortical blood flow caused by CSDs have been shown in humans (Olesen, 1998, Olesen et al., 1990). Hadjicani et al, recorded blood oxygen level-dependent (BOLD) signal changes typical of CSD, using fMRI in a migraine with aura patient (Hadjikhani et al., 2001). Interestingly, the alterations in blood flow do not always directly correlate to the presenting clinical features. Some patients that report not experiencing any aura symptoms none the less have significant events in the cortex (Woods et al., 1994, Denuelle et al., 2008, Thomsen et al., 1995, Olesen, 1998). This has led to the theory of "silent aura", that CSD still occur in migraine without aura patients but present with no clinical features.

Spreading depression also occurs in other neurological diseases including stroke, seizures, subarachnoid haemorrhage and traumatic brain injury (Mayevsky et al., 1996). Acute brain injury has been shown to increase the susceptibility to CSD (Strong et al., 2002). In traumatic brain injury patients, the presence of CSDs was a predictor of neurological outcome, with an increased risk of a worse outcome (Hartings et al., 2011, Woitzik et al., 2013). These conditions allow for direct recording using electrocorticography (which cannot be performed in migraine patients due to ethical

considerations) showing spreading depression in human tissue have electrophysiological properties comparable to those found in animal models of CSD (Hartings et al., 2013).

CSD is a massive cortical event that causes a huge depolarisation of cortical neurons and glial activation of the ipsilateral cortex. At the CSD wave-front, there is an increase in extracellular potassium (K^+), an influx of sodium (Na^+) and calcium (Ca^{2+}) to the cells and a release of glutamate (Fig. 15). It has long been proposed that the K^+ may be the initiating event (Grafstein, 1956), causing neuronal depolarisation, calcium influx from Cav2.1 channels (Tottene et al., 2011) leading to the release of glutamate and activation on NMDA receptors (Marrannes et al., 1988). NMDA activation causes further depolarisation and the start of a positive feedback loop which is the basis for the self-propagation of CSD. This initial excitatory wave is followed by a sustained inhibitory period of spontaneous firing in the cortex.

During CSD there is the release of a number of sensitising mediators, including ATP (Schock et al., 2007), glutamate (Basarsky et al., 1999), potassium and protons from neurons and glia (Haydon and Carmignoto, 2006) into the perivascular space, which have been suggested to sensitize primary trigeminal afferents in the perivascular space. Additionally, it has been suggested that perivascular nerves, potentially following their sensitisation, may release CGRP (Reuter et al., 1998) and nitric oxide (NO) (Read et al., 1997). It has been suggested that these mediators cause further neurogenic inflammation able to trigger the trigeminal afferent nociceptors that innervate the dural vessels and pia mater (Bolay et al., 2002). CSD has been shown to be able to enhance firing from the trigeminal ganglion (TG) and trigeminal nucleus caudalis (TNC) which remained elevated for up to an hour (Zhang et al., 2011), as well as, increase FOS expression (a marker of cellular activation) in laminae I and II of the TNC dorsal horn, which could be blocked by intravenous sumatriptan (Moskowitz et al., 1993). In migraine it is believed that CSD is initiated in the occipital cortex.

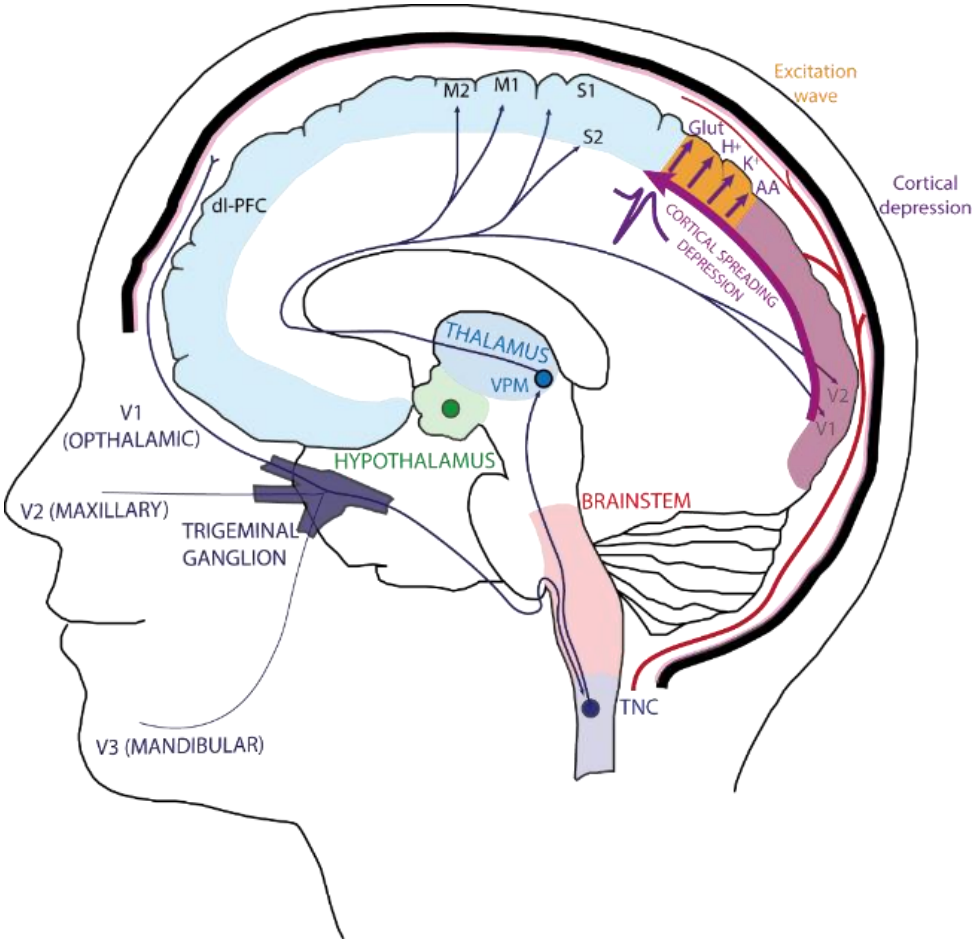

Figure 15: Diagram of cortical spreading depression wave arising from the occipital cortex, *showing the excitatory chest of the wave (orange) and the sensitising mediators released (Glutamate (glut), protons (H^+), potassium ions (K^+) and arachidonic acid (AA)) followed by cortical depression (purple). Adapted from (Holland et al., 2012).ventroposteromedial (VPM) thalamus*

The question as to why the cortex of the migraineous brain, and particularly the occipital cortex, is susceptible to these large depolarising events has led to the hyperexcitability theory. It suggests that an overall increase in activity of the cortex is what predisposes some migraineurs to migraine aura, with the theory expanding more to even suggest that this hyperexcitability of the cortex may underly the susceptibility of patients to migraine attacks (Welch et al., 1990, Denuelle et al., 2008, Woods et al., 1994).

There is much research in support of the cortical hyperexcitability hypothesis of migraine. Motor evoked potentials (MEP) Applied to the visual cortex several studies have shown a reduction of phosphene threshold in both migraine patients, both with and without aura (Aurora et al., 1998, Mulleners et al., 2001b, Gunaydin et al., 2006, Áfra et al., 1998,

Gerwig et al., 2005, Chadaide et al., 2007, Aurora et al., 2003, Aurora et al., 1999b) suggesting cortical hyperexcitability. However, one contradictory study did find an increase in activation thresholds in the visual cortex, suggesting hypoexcitability (Bohotin et al., 2003). The cortical excitability seen in the visual cortex may not be consistent cortex-wide as further studies using TMS have found either no difference (Gunaydin et al., 2006, Bohotin et al., 2003, Werhahn et al., 2000) or an increase in activation thresholds of the motor cortex (Áfra et al., 1998, de Noordhout et al., 1992, Bettucci et al., 1992). Several studies have found additional differences in cortical activation thresholds in migraine patients with and without aura (Gunaydin et al., 2006, Gerwig et al., 2005, Chadaide et al., 2007, de Noordhout et al., 1992, Chronicle et al., 2006, Aurora et al., 2003), suggesting the cortex of migraine with aura patients is more excitable than in migraine without aura patients. The contradictory results may be due to methodological differences, as not all studies are consistent in the diagnosis of migraine, including presence of aura and refractory status, ictal vs interictal testing and the type of TMS used (i.e.. Repeated measures vs paired pulse TMS).

Additionally, migraine patients (both episodic and chronic, with and without aura), have been found to have significantly increased levels of the excitatory neurotransmitter, glutamate in blood plasma and cerebrospinal fluid (CSF), both ictally and interictally (Siniatchkin et al., 2011, Cananzi et al., 1995, Martínez et al., 1993). Glutamate has long been known to be a trigger for spreading depressions (Harreveld, 1959), through NMDA receptors. NMDA receptor antagonists cause a complete block of electrically or chemically induced CSD (Gorji et al., 2001, Marrannes et al., 1988, Menniti et al., 2000) however CSD are not blocked when antagonists for AMPA Kainate receptors are applied (Lauritzen and Hansen, 1992, Krüger et al., 1999). Following successful prophylactic treatment, migraine patients plasma glutamate levels were significantly reduced compared with pre-treatment baselines (Ferrari et al., 2009).

The hyperexcitability theory has been lent further support by the efficacy of anti-epileptic medications such as topiramate (Storey et al., 2001) and sodium valproate (Hering and Kuritzky, 1992) as prophylactics for migraine. These drugs act primary on voltage gated sodium channels and $GABA_A$ receptors to decrease neuronal excitation (Shank et al., 2000). When treated with sodium valproate, the thresholds for phosphene production was

found to be increased in MA but not MwA patients (Mulleners et al., 2002) suggesting a reduction in cortical excitability. Similar findings have also been reported for metoprolol (Gerwig et al., 2012) and topiramate (Aurora et al., 2010) acting as a antagonist to kainate receptors (Andreou and Goadsby, 2011).

The causes of cortical hyperexcitability are likely multifactorial. Some of the genetic alterations that have been identified by genome wide association studies (GWAS) to occur in migraine patients, including the *MTDH* (Ligthart et al., 2011), *LRP1* (Chasman et al., 2011), *MEF2D* and *PHACTR1* (Freilinger et al., 2012) and especially the single point mutations found in familial hemiplegic migraine, on the *CACNA1A, ATP1A2* and *SCN1A* genes, suggest a genetic tendency for neuronal excitability.

Another suggestion is that hyperexcitability may stem from impaired inhibition of the cortex (Chronicle and Mulleners, 1994). Magnetic suppression of perceptual accuracy (MSPA) test has been used as a technique for investigating excitability differences between migraine patients and controls. In migraine patients there was a smaller reduction in accurate recall vs healthy controls (84.37 in chronic migraine and 57.41 in episodic migraine vs 19.19 in healthy controls) suggesting decreased inhibition (Aurora et al., 2007). MSPA has also shown impaired inhibition in migraine with aura but not migraine without aura patients (Chronicle et al., 2006). A similar technique, metacontrast visual masking, can also be used to investigate inhibitory actions in the visual cortex. Using metacontrast it was found that migraine with aura patients were less susceptible to visual contrast than migraine without aura patients or controls, suggesting impaired cortical inhibition. Additionally, this impairment was found to be corrected with sodium valproate (a centrally acting $GABA_A$ agonist) but not with other preventative treatments (Palmer et al., 2000). In the motor cortex it was found that the cortical silent period elicited by TMS was reduced in migraine with aura patients than healthy controls, leading to the conclusion that there is reduced cortical inhibition leading to increased excitability (Aurora et al., 1999a).

Finally, it should be remembered that migraine is not a static condition, it may not be as simple as a constant, global increase in activity, but there are ebbs and flows in cortical excitability (Smith et al., 1999, Andreou and Edvinsson, 2019). Migraine patients,

especially migraine with aura have shown greater variability in excitability between five different time points than healthy volunteers (Antal et al., 2006).

Previous work into the migraine treatment sTMS' actions on the cortex, found that sTMS can inhibit mechanical and chemical induction of CSD within the cortex when applied near the visual cortex (Andreou et al., 2016).

How this effect is achieved, has not been answered yet. The hypothesis had been that sTMS depolarises cortical neurons in a manner that they cannot be activated again by induction of CSD (Andreou et al., 2016). However, this has not been proven. A major limitation of the previous studies is that the actual actions of sTMS over cortical activity per se have not been investigated. To define this, I used *in vivo* recordings of the visual cortex and investigate how different sTMS intensities acutely alters tonic cortical neuronal firing.

The hyperexcitability of the cortex can be modelled by applying exogenous glutamate to stimulate individual cortical neurons being recorded from. This allowed me to observe the effect of a clinically relevant, acute application of sTMS on single excited cortical neurons. This was extended to include observations of the effect of sTMS in the presence of GABA antagonists. Thus, gleaning further insight into sTMS' interaction with the inhibitory control systems of the visual cortex.

Previous studies into sTMS' effect on CSD, focused on sTMS preventing the initiation of the CSD using mechanical (pinprick) and chemical (K^+) stimuli. In this chapter, I investigated, the change in the electrical stimulus required to generate a CSD, pre and post sTMS application, as well as in the presence of GABA antagonists. This is a more accurate method for investigating how the susceptibility of the cortex to CSD changes and if sTMS affects the threshold of activation. *In vivo* calcium imaging was also used to investigate the effect of sTMS application on the properties of the spread of the CSD wave once initiated. The use of both *in vivo* electrophysiological recording and calcium imaging allows recording of neuronal activity at the level of both individual neurons and the tissue as a whole.

The focus of this chapter is to investigate how sTMS application, as used in migraine, acutely affects activity in the cortex that it directly modulates. This includes sTMS'

interaction with; the principle cortical features of migraine, namely cortical hyperexcitability and CSD, as well as the excitatory and inhibitory systems of the cortex. By elucidating how sTMS acts directly on the cortex, a better understanding of sTMS mechanisms of action and how it ultimately results in improving migraine outcomes can be gleaned.

Chapter 2:

Acute direct effects of sTMS application on Cortical Neuronal Activity

Methods

Principles of extracellular electrophysiology

During neuronal action potentials, electrical potentials are produced via the distribution of ions across a cellular membrane (Hodgkin and Huxley, 1952). An electrode in close proximity to the neuronal cellular membrane can measure the voltage change in the tissue with high temporal resolution. Neuronal activity can be either spontaneous, stimulated from the introduction of drugs or evoked from a peripheral receptive field (if the recording cell is part of a somatosensory pathway).

a. Single-unit extracellular electrophysiology

Single-unit extracellular electrophysiology uses an insulated, metal electrode, described in greater detail below. As only the tip of the electrode is exposed the electrical signal recorded is from a localised vicinity, the activity from single unit or small group of units can be isolated. Additionally, the insulation also provides structural support so the electrode tip can be placed stereotaxically, easily penetrating through neuronal tissue, to record from specific nuclei. The metal core of the electrode is highly conductive with low impedance, producing high signal to noise ratio, beneficial for recording action potential spikes. The signal to noise can be further improved with a secondary recording electrode placed beneath the skin. The secondary electrode records general systemic physiological electrical activity which can then be subtracted from the activity recorded from the main electrode, leaving only the activity in the immediate vicinity of the electrode tip. The signal from the electrode does need to be amplified and filtered in order to discriminate action potentials. Details of the electronic setup used can be found in figure 19.

b. Direct current (DC) potentials:

CSDs causes widespread depolarisation of a large number of neurons as it travels in a wave across the cortex (Kraio and Nicholson, 1978). CSD's are characterised by a reduction in cortical spontaneous activity (Leao, 1944), changes in cortical blood flow, increases in cortical impedance (Van Harreveld and Ochs, 1957) and changes in the cortical steady state potential (DC shift) recordings (Leao, 1947). Cortical steady state potential recording can be used to record the net activity of the slow potential changes arising from CSD over a larger area of cortical tissue. A glass pipette filled with a

conductive solution (further details provided below), is placed into the superficial layers of the cortex. These electrodes have a lower electrical impedance making them suited to recording large net changes in cortical potential. As with the single-unit recordings, a secondary (silver-silver chloride) electrode is placed below the skin to remove systemic electrical noise from cortical activity at the electrode.

Principles of microiontophoresis

Microiontophoresis is combined with extracellular electrophysiological recordings. A recording electrode is prepared with two or more additional micropipette barrels, filled with an aqueous solution containing the desired drug in an ionic form. While recording electrical activity in the tissue, small quantities of a drug are ejected in the immediate vicinity of the electrode tip to observe its effects (Hicks, 1984).

The ionic charge of the drug molecules is controlled by adjusting the pH of the aqueous solution. An acidic solution donates proton (H^+) ion, causing it to have a positive charge. The opposite is true in a basic solution, losing a proton, making the drug molecule negatively charged. When an electrical current is applied to the solution, with the same charge as the molecule it causes it to be repelled out of the micropipette, i.e. a positive charge will expel a cation while a negative charge will expel an anion (Fig. 16). Although, the amount of drug expelled cannot be calculated, it is proportional to the charge delivered, thus the amount of drug can be titrated as necessary. Between ejections a small retaining current, of the opposite charge, is applied to the barrel to prevent leakage. To prevent the charge applied to the micropipette barrels transferring into the tissue being recorded, a central balance barrel is used. This micropipette barrel is filled with a neutral sodium chloride solution and has the opposite sum charge of all other barrels applied to it, thus balancing the current across the entire electrode so a zero-sum charge is applied to the tissue (Salmoiraghi and Weight, 1967). Typically, this technique is used for determining the effect of a substance on the activity of a neurone or muscle. Microiontophoresis has the advantage that only a single or small group of local neurons are affected, rather than introducing a substance systemically. Hence, it allows for multiple recording from a single animal.

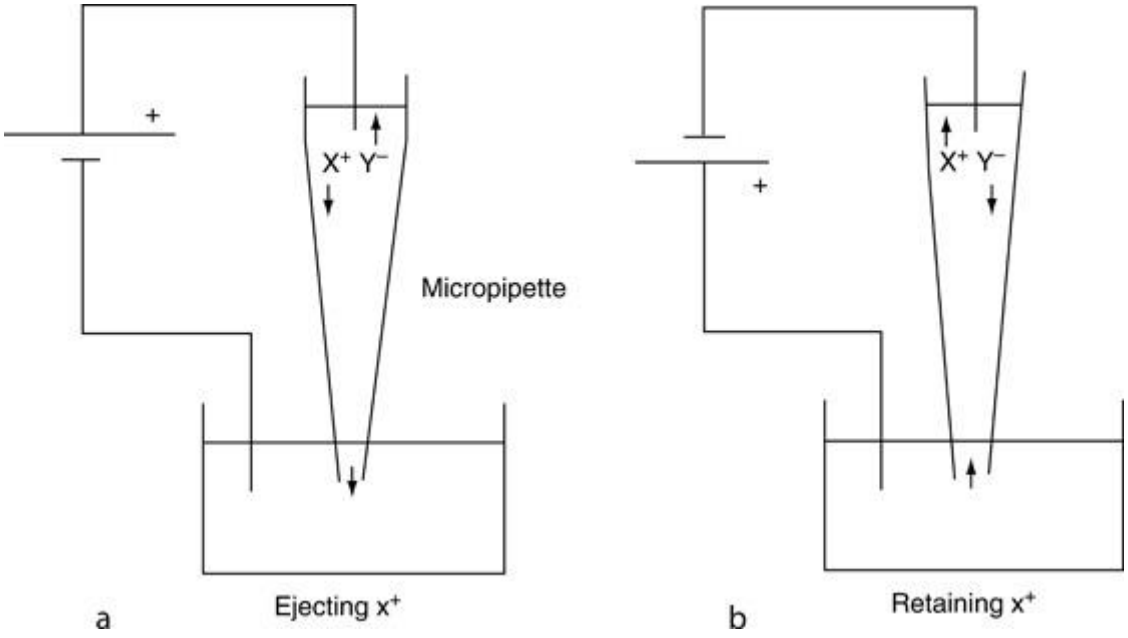

Figure 16: Principles of microiontophoresis *Schematic diagram of a micropipette that contains a salt X+Y−, showing the direction of current necessary to eject (a) and retain (b) the ion X+. (Hicks, 1984)*

Principles of GCaMP fluorescence imaging

Snap25-2A-GCaMP6s-D knock-in mice were used for cortical *in vivo* calcium imaging experiments. These mice have an ultrasensitive, slow variant calcium indicator (GCaMP6s) expressed down-stream of the synaptosomal-associated protein 25 (SNAP-25) coding region[1]. SNAP-25 with the attached GCaMP-6s marker is expressed pan-neuronally, in both the peripheral and central nervous systems, in the terminal boutons of neurons. During exocytosis, the change in membrane potentials causes the opening of voltage gated calcium channels in the pre-synaptic bouton and an influx of calcium ions. In addition to binding to synaptotagmin and causing the formation of the SNARE complex, calcium also binds to the GCaMP6s. The biding of calcium to GCaMP6s results in the expression of enhanced green fluorescence protein (eGFP) fluorescing at 509 nm. In the absence of calcium blinding there are very low levels of eGFP fluorescence (Fig. 17). Therefore, GCaMP6s fluorescence can be used as a marker of neuronal activity (Chen et al., 2013).

[1] A viral 2A oligopeptide sequence (T2A) is additionally inserted along with GCaMP6s to mediate ribosomal skipping.

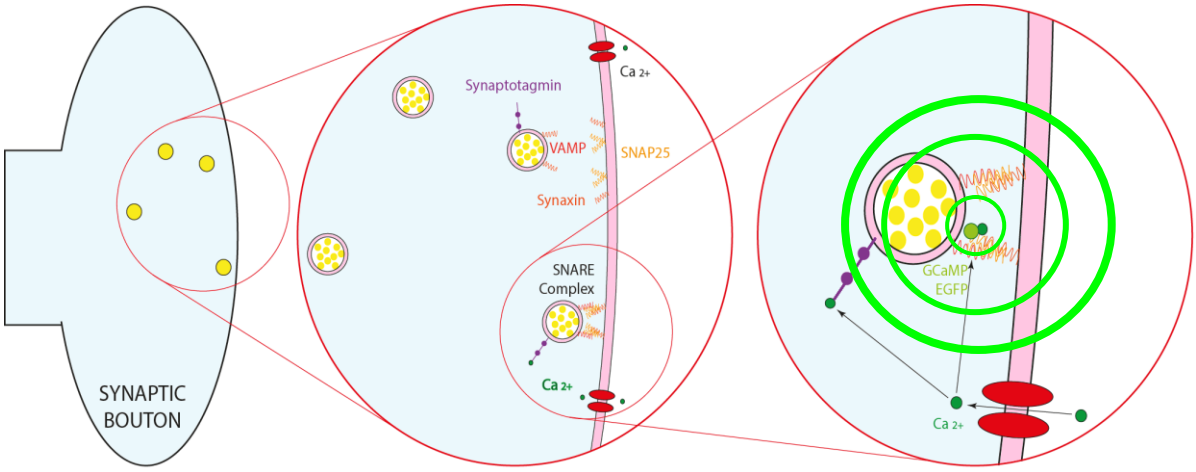

***Figure 17: Ultrasensitive calcium indicator GCaMP6 expressed downstream of SNAP25 protein in the synaptic bouton**, flouresces from calcium influx (during exocytosis), used as a marker of neuronal activation*

Materials and Equipment

sTMS

A bespoke rodent *in vivo* single pulse transcranial magnetic stimulator (sTMS) and coil were developed by eNeura Inc. (CA, USA) for use in scientific research (Fig. 18). The stimulator consists of a high-voltage power supply, energy-storage capacitor bank and circular magnet-wire coil of 11 mm diameter, kept in an insulated carbon fibre protector of 14 mm diameter for minimising heat produced by the coil during TMS. The coil is connected to the stimulator via a rise-time adjustment box set at 170 µs. This mirrors the time rise of the sTMS clinical device for treatment of migraine. The stored energy in the capacitor bank rapidly discharges a high current pulse passing through the coil. This creates an individual, transient, monophasic magnetic pulse of 170 µs rise time and a pulse width of 360 ms. The capacitor can be re-charged, this process lasts ~5 - 60 s, depending on the voltage supply (100 - 600 V, Table 4). Pulse intensity is variable giving a range of magnetic pulses up to ~1.1 T when triggered, measured using a Gaussmeter at 5 mm distance from the coil. A calibrated pickup coil and pulse detection circuit allows the magnetic pulse to be displayed on an oscilloscope and the pulse characteristics recorded in a data management system. Pulse intensity is variable giving a range of magnetic pulses up to ~1.1 T when triggered.

The maximal voltage stored by the capacitors is 680 V, 600 V was decided as the maximum to provide a safety margin and because it is equivalent to the output from the human device (~ 1.1 T)

***Table 4**: **Magnetic field strength in Tesla (T)** as measured by Gaussmeter at a 5 mm distance of the coil, connected to 170 µs tap on risetime adjustment box*

Voltage (set point)	***Tesla output***
50	0.093
100	0.193
200	0.377
300	0.565
400	0.744
500	0.924
600	1.103

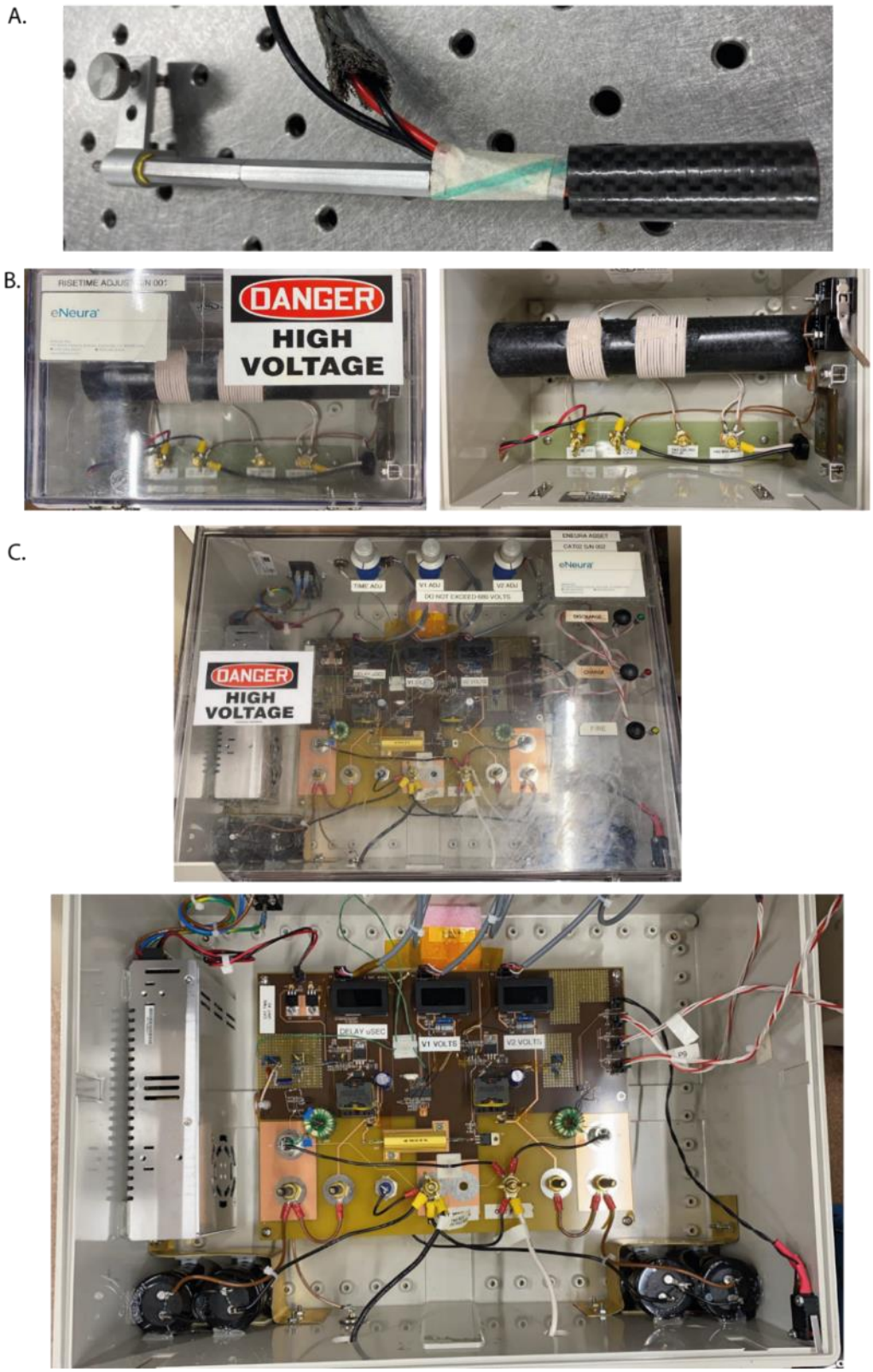

Figure 18: Bespoke rodent in vivo single pulse transcranial magnetic stimulator (sTMS) and coil. *(A) 11 mm circular magnet-wire coil, insulated by 14 mm carbon fibre protector (B) External and internal images of risetime adjuster set at 170 µs (C) External and internal images of stimulator high-voltage power supply, energy-storage capacitor bank*

Recording electrodes and electrophysiological equipment

Single cell electrophysiological recordings

Single cell neuronal activity was recorded using a Tunglass-1 glass-insulated tungsten microelectrode (W1011, Kation Scientific LLC, USA), which consists of a tungsten wire within a borosilicate glass sheathing (OD 40 µm) with an exposed tip of 10 – 30 µm (Impedance @ 1 kHz: 0.8 – 2.8 MΩ). The recording electrode was attached to a Digitimer NL100 head-stage amplifier (CED, UK). Signal from a second reference 23 G needle (B-in) placed in the temporalis muscle was also fed into the head-stage, which was ground through connection to the experimental rig. The signal from the NL100 head stage was fed into Digitimer Neurolog NL900D (CED, UK), amplified by A.C PreAmp (NL104) at a gain of 100 and the B-in signal subtracted from the main recording signal. 50/60 Hz noise was filtered with a noise eliminator (Humbug, Quest scientific, Canada), the signal was further filtered through a NL125, NL126 module between 500 and 1,500 Hz, the filtered signal was amplified by AC/DC Amp (NL106) at a gain of approximately 500, before the signal was converted from analogue to digital by a Digitimer Micro 1401-3 (CED, UK) and displayed on Spike2 (version 7 Software, CED, UK) on a personal computer (Fig. 19).

Microiontophoresis

Microiontophoresis studies used a carbostar-7s recording/iontophoresis combination microelectrode (E1073, Kation Scientific LLC, USA). These are 7 glass micropipette tubes incorporating a glass coated carbon fibre recording electrode (Impedance @ 1 KHz: 0.4 – 0.8 MΩ) with an exposed tip length of 15 – 20 µm and a diameter of 2 – 3 µm. Single cell neuronal recordings were filtered and amplified as described above and displayed on Spike2 on a personal computer. When the electrode was not in use the electrode tip was placed into a 0.9 % saline (PHA/SAL/003, B. Braun, Germany) bath to avoid microcrystal formation at the tip.

Micropipette barrels were connected to the micro-iontophoresis current generator (Dagan 6400, Dagan Corporation, USA) via 0.25 mm Teflon coated silver wires (AGT1010, World Precision Instruments, USA) placed into the aqueous solution in each micropipette

barrel. Output from the Dagan 6400 was monitored through outputs to the Digitimer 1401 and displayed on Spike2 (version 7 Software, CED, UK) on a personal computer (Fig. 19).

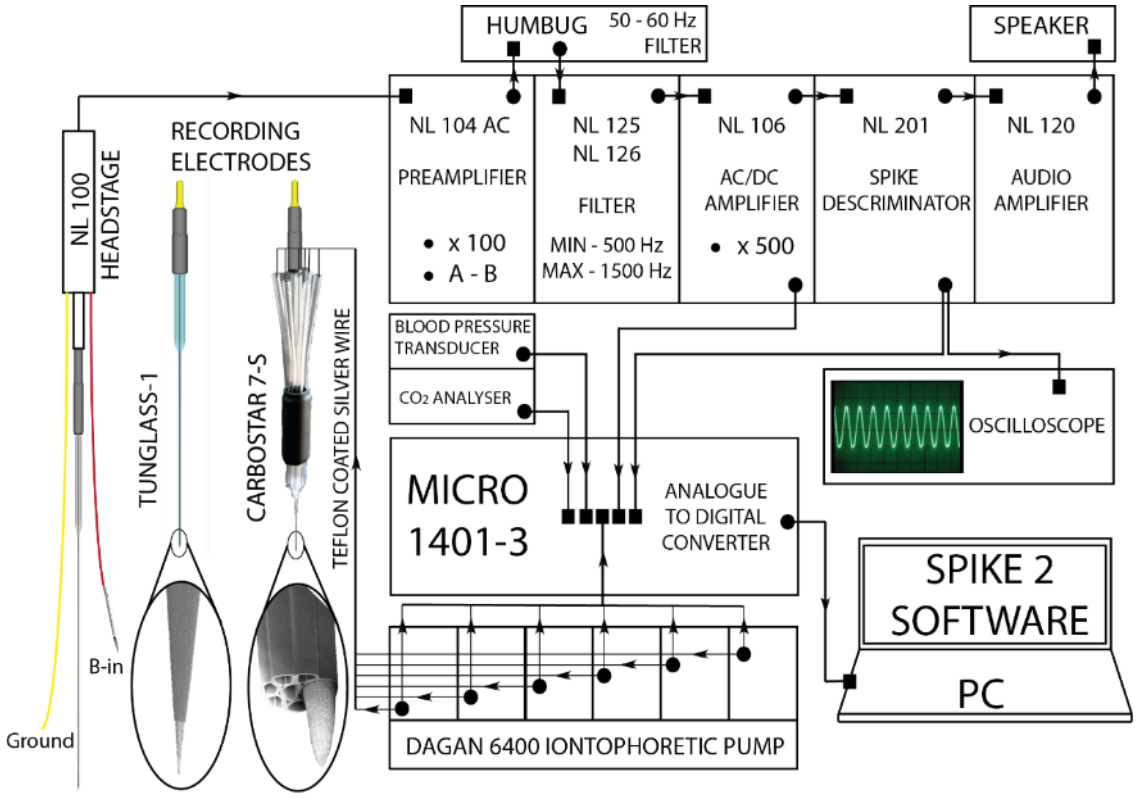

Figure 19: Schematic diagram of the recording electrode equipment setup used in the electrophysiological experiments

Drugs used in microiontophoresis studies

All drugs used in microiotophoresis experiments were dissolved in distilled deionised water. The pH was tested using either pH meter (240, Corning, USA) or pH indicator strips (pH 2.0 – 9.0) (HC568406, Millipore, USA). The pH was adjusted by adding small amounts of either 0.1 M hydrochloric acid (J/4270/17, Fisher Scientific, UK) or 0.1 M sodium hydroxide (28245.265, VWR Chemicals, Belgium) until the optimum iontophoretic pH was reached. To avoid blocking of the tip, micropipette barrels were filled solutions at room temperature using a Millex Filter Unit with a hydrophilic Durapore (PVDF) membrane (pore size 0.22 µm) (Z227501, Sigma-Aldrich, UK) and a 34 G/67 mm microfil pipette filler (MF34G, World Precision Instruments, USA) at least

1 hour prior to use. Additionally, filaments within the micropipette barrels allowed self-filling of solutions to the tip. Anions (-) were retained in the barrels with small positive currents (5 - 10 nA) while cations (+) were retained with small negative currents (-5 – 10 nA). Ejection currents were in the opposite charge to the retaining currents and titrated for the cell being recorded, but typically between 30 – 90 nA.

A central balancing micropipette barrel was filled with 0.15 M Sodium Chloride (S9888, Sigma-Aldrich, USA) at pH 7.4. Up to 3 micropipette barrels were filled with 200 mM anionic solution of L-Glutamic acid monosodium salt (G1626, Sigma Aldrich, USA) at pH 8.0. One micropipette barrel was filled with the $GABA_A$ antagonist, (-)-bicuculline methochloride (0131, Tocris, UK) in a cationic solution of 0.1 M and pH 3.5. Bicuculline was retained at -5 nA and ejected at +45 nA. One barrel was filled with the $GABA_B$ antagonist, 2-Hydroxysaclofen (0245, Tocris, UK) in an anionic solution of 0.1 M and pH 9.0. 2-Hydroxysaclofen was retained at 5nA and ejected at -60 nA (Table 5).

For marking the recording site one micropipette battel was filled with 2.5% Pontamine Sky Blue (C8679, Sigma-Aldrich, USA) in 0.1 M Sodium acetate (S2889, Sigma-Aldrich, USA). Following the completion of the experiment pontamine sky blue was microiontophoresed to stain the recording site, using an iontophoresis generator (ION-100T Cornerstone, USA) (PS100, Dagan Corporation, USA) which provided an ejection current of -1990 nA for approximately 15 – 20 minutes.

Table 5: Properties of Microiontophoretic drugs

Drug	*Concentration*	*pH*	*State*	*Retaining Current*	*Ejecting Current*
L-Glutamic acid monosodium salt	0.2 M	8.0	Anionic	+ 5 nA	- 80 nA
(-)-bicuculline methochloride	0.1 M	3.5	Cationic	- 5 nA	+ 45 nA
2-Hydroxysaclofen	0.1 M	9.0	Anionic	+ 5 nA	- 60 nA
Pontamine Sky Blue	2.5 %		Anionic	+ 5 nA	- 1990 nA

Cortical steady state DC potential recordings

CSD was used as a model of migraine aura. Induction of CSD was mainly monitored by cortical steady state potential (DC shift) via a 1.5 mm borosilicate glass microelectrode (Warner Instruments, USA) containing 3 M NaCl (S9888, Sigma-Aldrich, USA) and a tip diameter of 1 – 2 µm. The microelectrode was coupled to a Ag/AgCl reference electrode and connected to an NL102G head stage signal was fed into a coupled Bridge Balance and DC Preamp (NL102), the signal was then passed through a Filter (NL 125) set to "DC" and amplified through an AC-DC Amp (NL106) at a gain of 400, before being fed into a Digitimer 1401 and displayed on Spike2 (version 7 Software, CED, UK) on a personal computer (Fig. 20).

Laser Doppler recordings

As a secondary measure, blood flow changes caused by the CSD wave, were monitored via laser doppler (Moor Instruments) using a 1.5 mm (OD) optical probe (VP3, Moor Instruments, USA). The probe was attached to a laser doppler perfusion and temperature monitor (DRT4, Moor Instruments, USA) which was in turn fed into Digitimer 1401 and displayed on Spike2 (version 7 Software, CED, UK) on a personal computer (Fig. 20).

Stimulating electrodes

Electrically induced cortical spreading depression

CSD was induced electrically using a concentric bipolar stimulating electrode (FHC, USA) applied superficially to the occipital cortex. Electrode was attached to Constant Current Isolated Stimulator (Model DS3, Digitimer, USA) (Fig. 20).

Superior sagittal sinus (SSS) electrical stimulation

Platinum wire stimulating electrodes with horizontal tips (2 mm approx.) and approximately 1 mm gap between the contacts were used to stimulate trigeminal afferents innervating the SSS. The stimulating electrode was connected to a Dual Output Square

Pulse Stimulator (S88, Grass Instruments, USA) via a stimulus isolation unit (S1U5B, Grass Instruments, USA) (Fig. 20).

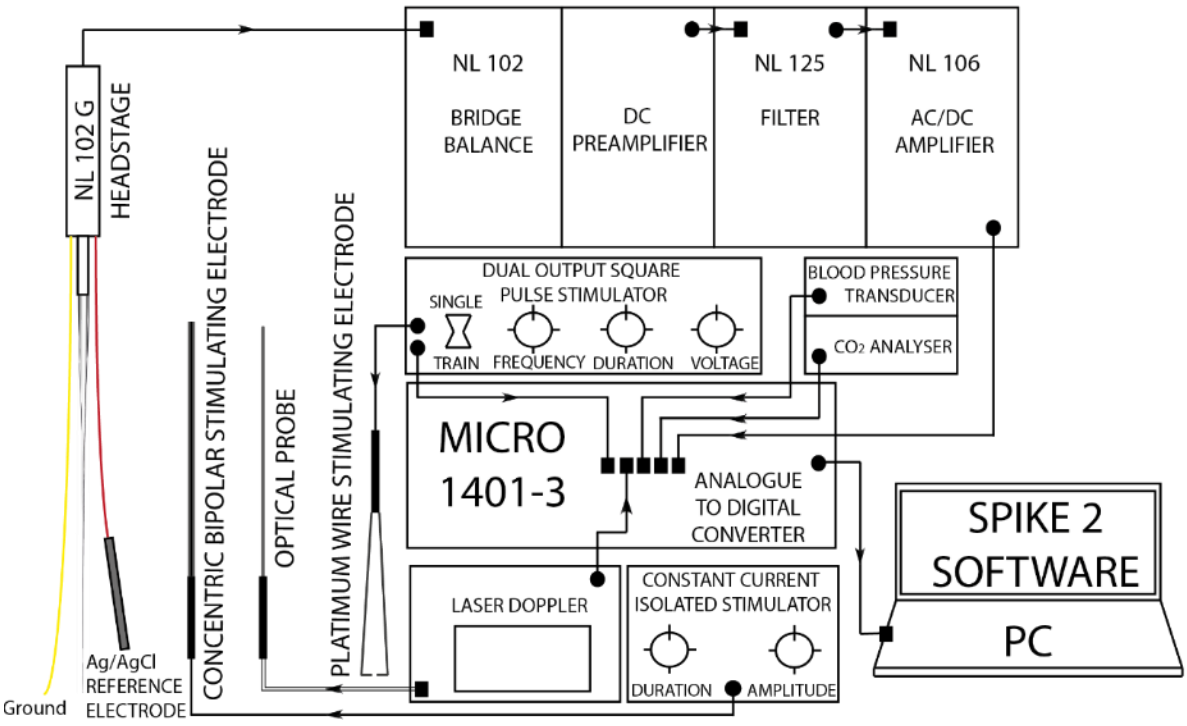

***Figure 20: Schematic diagram of additional equipment used in the electrophysiological experiments**, including; stimulating electrode setup, DC-shift setup and laser doppler setup utilising an optical probe*

Animals and ethical approvals

All experiments were performed in accordance with the United Kingdom Home Office Animals (Scientific Procedures) Act (1986). Experiments were carried out in adult male Sprague Dawley rats (*N* = 115, 250 - 350 g; Charles River, UK), in adult Snap25-2A-GCaMP6s-D mice (*N* = 17; 11 males, 6 females; 25 - 35g; Jackson Laboratory, Bar Harbour, USA) and in adult C57BL/6 mice (*N* = 5; 3 males, 2 females; 25-35 g; Jackson Laboratory, UK). Both rats and mice were housed on a 12/12-hour light/dark cycle with food and water available *ad libitum*. All experiments were performed under general anaesthesia and terminated by anaesthesia overdose and/or cervical dislocation.

Surgery

Rats

General anaesthesia was induced with intraperitoneal injection of 60 mg.kg-1 pentobarbital sodium (Merial, UK). Supplementary anaesthesia was maintained with continuous intravenous infusion of pentobarbital (12-15 $mg.kg^{-1}.h^{-1}$). A tracheotomy was performed to permit ventilation of the animal and end-tidal expired CO2 was monitored and maintained between 3.5 - 4.5% (Capstar-100, CWE). The left femoral vein and artery were cannulated to allow for constant intravenous infusion of anaesthetic and monitoring of blood pressure, respectively. Adequate anaesthesia was gauged by the absence of toe pinch withdrawal and eye-blink reflexes and gross changes in blood pressure. Core temperature was monitored and maintained near 37°C using a homoeothermic blanket system (TC-1000, CWE). The animal was then fixed on a non-magnetic stereotaxic frame (Kopf instruments). Craniotomies were performed and dura mater incised to expose the occipital and parietal cortex for electrophysiological recordings/stimulations. The sTMS coil was held on a Kopf mounted holder and placed over the visual cortex ~5 mm from the surface of the skull.

Mice

Snap25-2A-GCaMP6s-D and C57BL/6 mice were used for *in vivo* imaging experiments. General anaesthesia was induced through the intraperitoneal injection of 0.3 ml urethane (12.5% in saline) and additional doses were titrated based on the depth of anaesthesia which was assessed by hindlimb withdrawal to toe pinch and corneal reflex activity, until surgical depth was achieved. Core body temperature was maintained at 37°C through a homeothermic mat with an associated rectal probe (Harvard Apparatus). A tracheal catheter was installed to ensure maintenance of a clear airway and the mice breathed spontaneously. The skull surface was exposed and the parietal bone on one side was secured to a custom-made head mount stage, for stability and alignment, using orthodontic acrylic resin and cyanoacrylate glue (Lang, Wheeling), leaving the other side accessible. A cranial window was drilled between bregma and lambda to expose the cortex. The window was sealed using a glass coverslip and Vaseline grease.

Spontaneous neuronal activity recordings

Extracellular spontaneous activity from single units in the visual cortex was recorded using a glass-insulated tungsten microelectrode (Kation Scientific, USA) with an impedance of ~1 MΩ. For cortical recordings the electrode tip was lowered approximately 800 µm into the occipital cortex, approximately 5 mm anterior from the sTMS electrode (Fig. 21A). Signals were amplified to a gain of 5000 (Neurolog, Digitimer, UK; NL104, NL106), fed through a Humbug noise eliminator (Quest Scientific, Canada) and bandpass filtered (500 - 4000 Hz, NL125/NL126). The conditioned signal was displayed on analogue and digital storage oscilloscopes and digitised for storage on a computer using a Micro 1401-3 with Spike 2 software (CED, UK).

Induction and recordings of cortical spreading depression

A small cranial window was drilled over the frontal cortex and another prepared over the visual cortex. The latter was used to induce CSD as described below. Cortical steady potential was recorded via 1.5 mm borosilicate glass microelectrode with a tip diameter of 1 - 2 µm containing 3 M NaCl, inserted to a depth of 50 - 100 µm in the frontal cortex. The microelectrode was coupled to a reference Ag/AgCl electrode placed in contact with exposed neck muscle. The signal was amplified (NL102G head-stage, NL102 DC amplifier) and displayed on a computer. Warm mineral oil was used to prevent exposed areas drying.

CSD was induced electrically, using a constant current isolated stimulator (DS3, Digitimer) with a concentric bipolar stimulating electrode (tip diameter 25 µm, FHC, USA) inserted to a depth of ~100 µm into the visual cortex. Amplitude and duration of the electrical stimulating pulse was increased until a CSD wave was triggered in order to determine the induction threshold.

Microiontophoresis

Seven-barrelled carbon-fibre electrodes were used to deliver drug solutions and a dye for marking recording sites, using a microiontophoresis current generator (Dagan 6400,

Dagan Corporation, USA), while simultaneously recording single unit neuronal activity. Micropipette barrels were filled with: L-glutamate, 200 mM pH 7.4 (Sigma-Aldrich, UK); (-)-bicuculline methochloride, 100 mM, pH 3.5 (Tocris Cookson, UK); 100 mM 2-hydroxysaclofen ($GABA_B$ antagonist; Santa Cruz Biotechnology Inc., Dallas, US) at pH 9.0, pontamine sky blue (PSB) dye, 2.5% w/v in 100 mM sodium acetate, pH 6.5 (BDH Laboratory Supplies, UK); NaCl, 1.0 M, pH 7.5 for automated current balancing. Microiontophoretic barrels had resistances of 20-150 MΩ. L-glutamate was ejected as an anion (10 - 90 nA) and retained with small positive currents; bicuculline and PSB were ejected as cations and retained with small negative currents. Neurons were excited by the programmed ejection of L-glutamate in timed pulses. The ejection (10 - 20 sec) and rest (10 - 20 sec) periods were adjusted for each cell to produce a sustainable and reproducible response with firing rates of typically 10 - 50 spikes.sec^{-1}. In the experiments with bicuculline, the drug was applied at 45 - 90 nA. Spontaneous and evoked neuronal activity was simultaneously recorded via the carbon fibre recording electrode and signals were filtered, amplified and processed as above. PSB was ejected at 2 µA at the end of each experiment to mark the recording site. Brains were collected upon termination of the experiment, sliced using a microtome and sections were examined under a microscope for the presence of a PSB spot. Where a PSB spot could not be identified, the electrode track was traced to identify the recording site.

In vivo cortical imaging

Snap25-2A-GCaMP6s-D or C57BL/6 mice were positioned under an Eclipse Ni-E FN upright confocal/multiphoton microscope (Nikon, UK) using a 4x dry objective. Images were acquired at a frame rate of between 0.25 and 2 Hz, depending on experimental requirements and signal strength. To visualise blood vessels, C57BL/6 mice were given injected via the tail vein with 0.05 ml of Dextran tetramethylrhodamine, (MWt. 70,000, lysine fixable solution, 10 mg.ml-1, Thermo-Fisher Scientific, UK). To obtain confocal images a 488 nm Argon ion laser was used, and a Coherent Chameleon II laser tuned to 920 nm for multiphoton imaging. GCaMP signal was imaged at 500 – 550 nm, Dextran signal at 570 - 620 nm. Time series recordings were taken with a fully open pinhole for maximal signal collection.

Experimental protocols

Spontaneous cortical neuronal activity and sTMS

To assess the actions of sTMS on ongoing cortical activity, a stable baseline of spontaneous activity was recorded for at least 20 min from neurons in the visual cortex. Two sTMS pulses of intensity between 0.2 - 1.1 T were then delivered, charging the stimulator at 100 V intervals up to 600 V (N = 6/group; total N = 36). Spontaneous activity was recorded for up to 90 min post-sTMS (Fig. 21A & B).

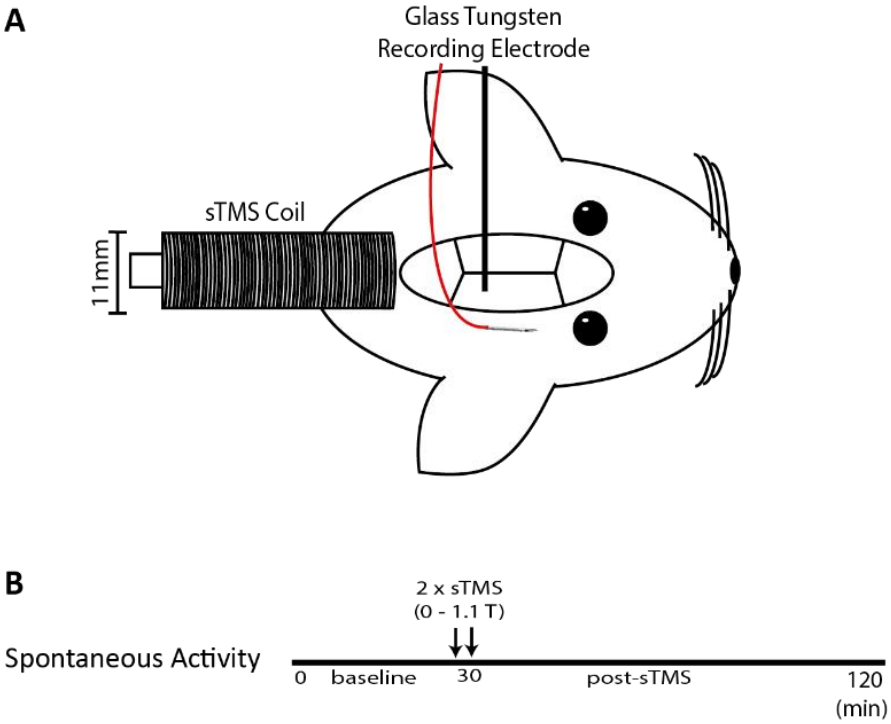

Figure 21: Acute sTMS application on spontaneous cortical activity *(A) Experimental setup in the rat model showing sTMS application site and electrode position (B) Diagram illustrating the experimental protocol employed in effect of sTMS on spontaneous cortical activity*

L-Glutamate evoked excitability and sTMS

To assess the actions of sTMS on cortical glutamatergic excitability, microiontophoresis of L-glutamate was used to excite cortical neurons within the visual cortex. At least 5 minutes under baseline conditions were recorded. Two sTMS pulses (0 T sham stimulation or ~1.1 T active stimulation) were then applied to the visual cortex. The train of L-glutamate pulses was continued for at least another 30 minutes post-sTMS application (Fig. 22A Bi).

To evaluate the influence of GABAergic activation as a potential mechanism of action of sTMS, in a separate set of experiments, either the $GABA_A$ antagonist bicuculline or the $GABA_B$ antagonist 2-Hydroxysaclofen was constantly microiontophoresed along with L-Glutamate ejected in pulses. As previously, at least 5 min of L-Glutamate evoke-activity were recorded in the in the presence of bicuculline, before two sTMS pulses (1.1 T or 0 T) were applied to the visual cortex. The train of glutamate pulses and the constant GABA antagonist ejections was continued for at least another 30 minutes post sTMS application (Fig. 22Bii).

Upon completion of the experiment, PSB was ejected to mark the recording location. Brains were removed and stored in 4 % paraformaldehyde until they were cut at 30 µm sections to identify the dye spot.

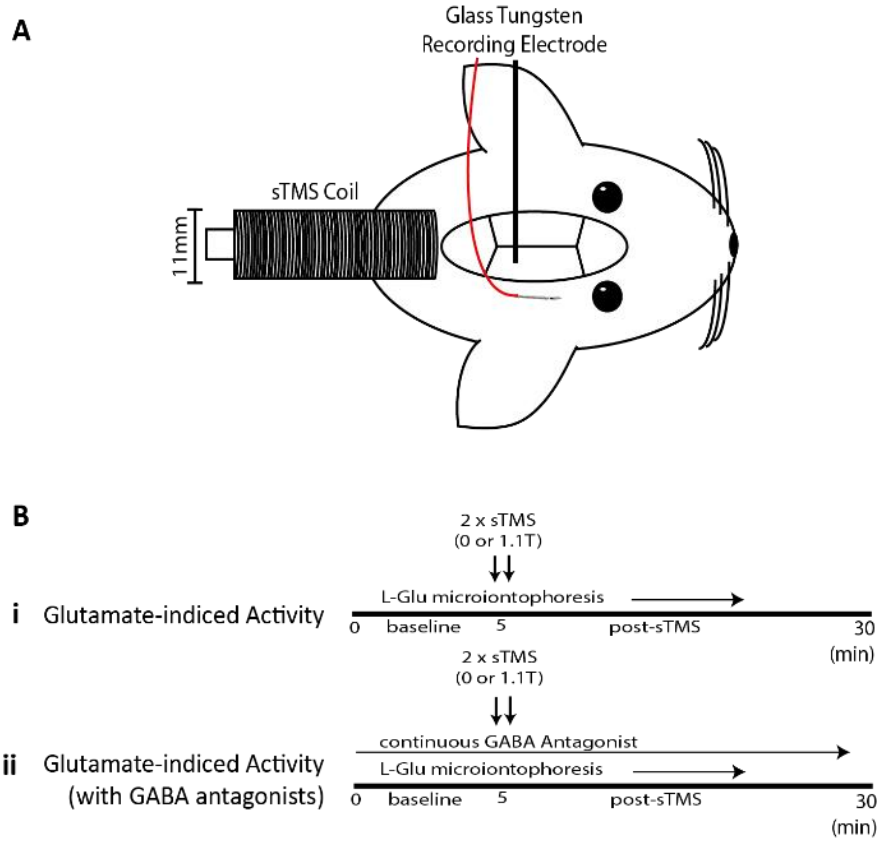

Figure 22: Acute sTMS application on glutamate induced cortical activity *(A) Experimental setup in the rat model showing sTMS application site and electrode position (B) Diagram's illustrating the experimental protocol employed in effect of sTMS on glutamate induced cortical activity and effect of sTMS on glutamate induced cortical activity in the presence of GABA antagonists*

Electrically induced CSD and sTMS

To identify if sTMS increases the threshold of activation for CSD induction, the migraine aura model of electrically induced CSD was utilised (Fig. 46A). Animals were pre-treated with two sTMS pulses at 0 T (N = 8) or ~1.1 T (N = 8). Afterwards, amplitude and duration of the electrical stimulation were increased until a CSD wave was triggered, Briefly, CSD threshold was found by increasing duration (50, 100, 200, 400 µs) and amplitude (0.5, 1, 2, 3, 4, 5 mA) of electrical stimulus .The CSD induction threshold was found in; animals pre-treated with two sTMS pulses (~ 0 T or 1.1 T; N = 8 per group) over the visual cortex before induction of a CSD, in animals that randomly received topical application of GABA antagonists bicuculline (N = 5), or 2-hydoxysaclofen (N = 5) over the visual cortex prior to receiving two sTMS pulses (~ 1.1 T). (Fig. 46Bi-ii).

The applied charge was calculated from the electrical stimulation parameters using the following formula;

$$Q = I \; x \; t$$

With Q the charge in microcoulombs, I the current in milliamps and t the time in microseconds (µs) (Table 6).

Table 6: Conversion table for threshold charge (mC) to initiate CSD

	0.5 mA	**1 mA**	**2 mA**	**3 mA**	**4 mA**	**5 mA**
50 µs	25 mC	50 mC	100 mC	150 mC	200 mC	250 mC
100 µs	50 mC	100 mC	200 mC	300 mC	400 mC	500 mC
200 µs	100 mC	200 mC	400 mC	600 mC	800 mC	1,000 mC
400 µs	200 mC	400 mC	800 mC	1,200 mC	1,600 mC	2,000 mC

In a separate set of animals (N = 8), the CSD threshold was first established under baseline conditions. Sixty minutes later, two sTMS pulses (~1.1 T) were delivered over the visual cortex and induction of CSD using the same baseline stimulating parameters was attempted every 30 min up to 2 hour post-sTMS stimulation. If CSD could not be induced using the pre-sTMS intensity, the threshold was re-examined by increasing stimulation current and duration until a CSD wave was induced (Fig. 23Biii).

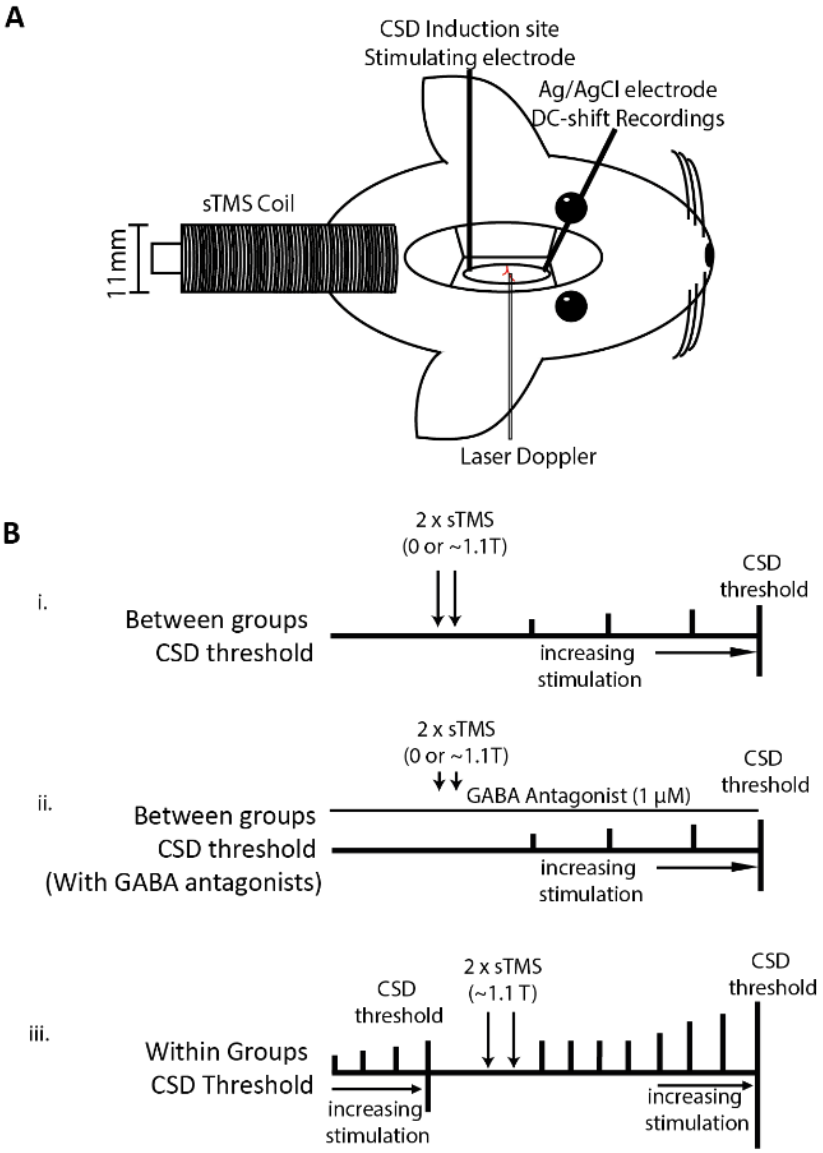

Figure 23: Effect of sTMS on cortical spreading depression electrical induction thresholds *(A) Experimental setup in the rat model showing sTMS application site, stimulating and recording electrode positions (B) Diagram's illustrating the experimental protocol employed in effect of sTMS on cortical spreading depression electrical induction thresholds and cortical spreading depression electrical induction thresholds in the presence of GABA antagonists.*

In vivo cortical imaging

Given the stimulus artefact produced by the sTMS machine and recorded in electrophysiological experiments, it remains unclear whether sTMS per se excites cortical neurons at these intensities. To answer this question *in vivo* calcium imaging in Snap25-2A-GCaMP6s-D mice was employed to visualise the immediate effect of sTMS on cortical calcium uptake as a marker for cortical activity. Baseline recordings of the

GCaMP signal were taken from the visual cortex for 5 min before a sTMS pulse (~1.1 T) was applied caudal to the cortical window. Image recording was continued for at least a further 5 min. As a positive control of neuronal excitation CSD was induced in the cortex. Due to practical limitations of the testing chamber the setup for previously used electrically induced CSD could not be used, thus CSD was mechanically induced via needle prick stimulation of the cortex, this is a reliable and well documented method of inducing CSD in animal models (Andreou et al., 2010b). A 30 G needle was inserted to a depth of ~1 mm into the cortex at the caudal aspect of the cortical window (Fig. 24A & B).

To assess if sTMS can induce changes in the cortical vasculature, C57BL/6 mice were injected with Dextran dye and the diameters of two blood vessels per animal were measured. Baseline measurements were recorded for at least 5 min and following 2x sTMS pulses at ~1.1 T, applied as before. As a positive control of blood vessel diameter changes a CSD was induced before completion of the experiment by inserting a 30 G needle ~1 mm into the cortex through a gap left at the rear of the cortical window for this purpose (Fig. 24A & B).

In a separate set of experiments, we sought to assess the effect of sTMS on the CSD parameters, when eventually a CSD could be induced following active sTMS stimulation. Baseline GCaMP signal activity was recorded through the cortical window for 15 minutes before 2x 1.1 T sTMS was applied to the visual cortex. Following at least a 10 minute break induction of a CSD was attempted via mechanical stimulation of the cortex with a 30 G needle as previously. When a CSD was induced, cortical activity was recorded for 30 minutes post-CSD wave.

In half of the animals who previously received 0 T sham sTMS stimulation, 2x 1.1 T sTMS was applied to the visual cortex at least 30 minutes post- first CSD induction. Following at least a 10 minute break a second CSD induction was attempted via mechanical stimulation of the cortex with a 30 G needle in all animals. When a CSD was induced, cortical activity was recorded for 30 minutes post-CSD wave.

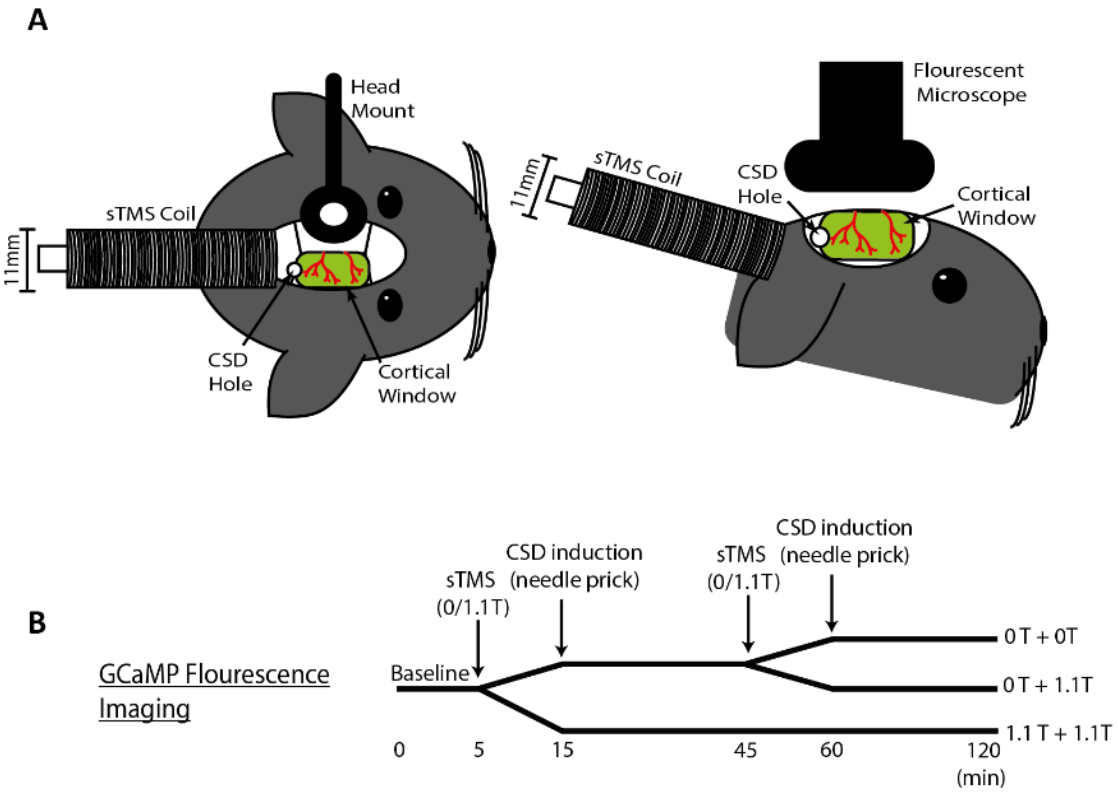

Figure 24: Acute sTMS application on cortical calcium activity *(A) Experimental setup in the rat model: horizontal and sagittal views, showing TMS application site and cortical viewing window (B) Diagram illustrating the experimental protocol employed in in vivo cortical calcium imaging,*

Data Analysis

Spontaneous neuronal activity studies

Spontaneous activity was recorded in Hz in cumulative histograms in Spike2Data (.smr) files, which were analysed using Spike2 (version 7, Cambridge Electronic Design, UK). Spike sorting was introduced to refine the isolation of neuronal spikes from background noise when required. The 'Edit Wave Mark' tool in Spike 2 was used to identify and filter waveforms to only those produced by neuronal activity (Fig. 25).

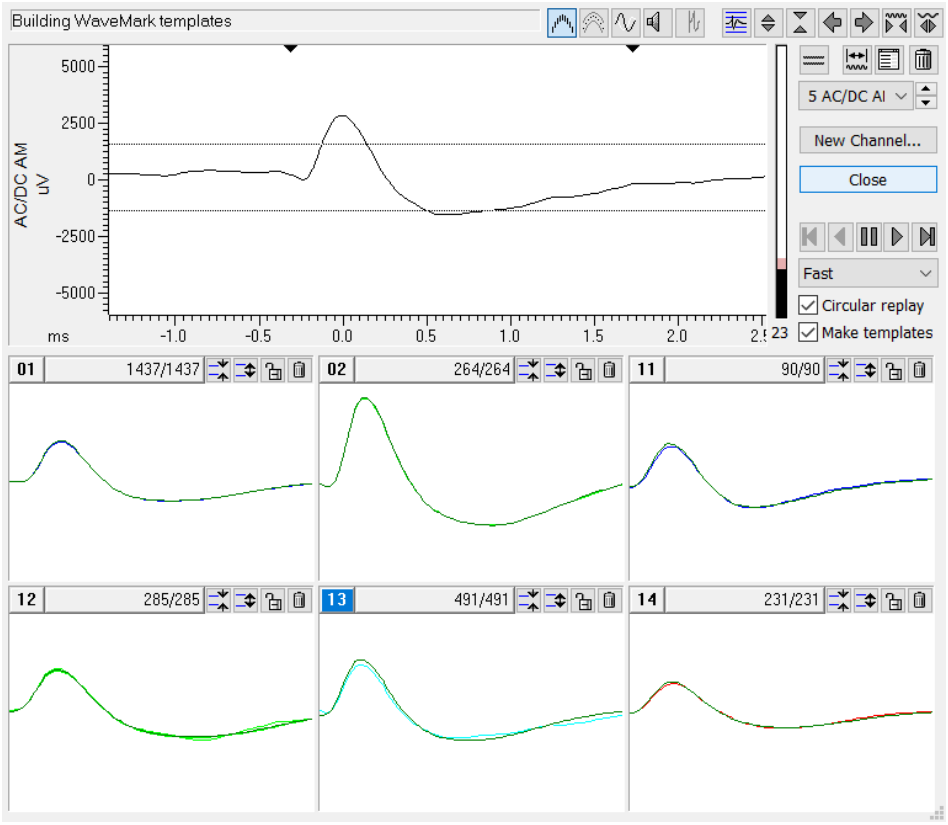

Figure 25: Example spike sorting using Wavemark template tool in Spike2 to identify and filter waveforms

For each unit, firing rate in spikes.sec^{-1} was averaged in 5 min intervals for 20 min pre-sTMS and up to 90 minutes post-sTMS. Repeated measures analysis of variance (rmANOVA) was computed with two factors: sTMS and Repeats, to determine stimulation effects on spontaneous neuronal activity, followed by Student's paired t-test. When the assumption of sphericity with regards to the factor of Repeats was violated, adjustments were made for the degrees of freedom and P values according to the Greenhouse-Geisser correction. All data were expressed as a mean percentage of baseline ± SEM and significance assessed at the $P < 0.05$ level (SPSS version 23.0; SPSS Inc., USA).

L-glutamate evoked activity studies

Neuronal firing in response to ~5 - 10 second microiontophoretic ejection of glutamate was analysed as cumulative rate histograms in 1 second bins using Spike2 (Fig. 26) (version 7, Cambridge Electronic Design, UK). A stable baseline of at least 5 subsequent

and comparable glutamate-induced firing periods were taken, multiple pulses were averaged to account for the natural variability in neuronal firing.

Digital output of the current applied to the individual barrels of the micro-iontophoresis current generator were displayed on separate channels in Spike2 (version 7 Software, CED, UK). These channels were used as a guide for the start and end of the glutamate-induced firing rate. This was necessary, as although the ejection of glutamate from the electrode could be precisely controlled by application of current, the chemical action on the cell persisted beyond the ejection duration. The firing time during the baseline period was then used for analysing subsequent pulses to maintain consistency throughout the experiment. Mean firing rate during the ejection period was averaged over a 5 minute periods to avoid variations of the responses of a cell between individual pulses.

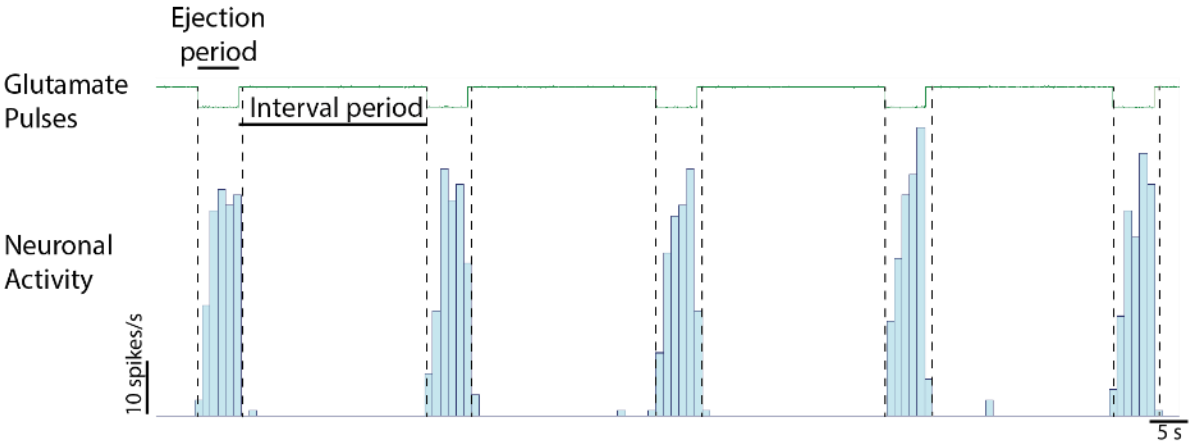

Figure 26: Example cumulative histogram of 5 subsequent neuronal responses to glutamate pulses used as baseline

The neuronal response to glutamate following application of either sTMS or a sham control were compared to the baseline neuronal activity. Changes to glutamate-induced neuronal activity was compared; (i) with glutamate induced activity alone (ii) in the presence of Bicuculline ($GABA_A$ antagonist) and (iii) in the presence of 2-Hydroxysaclofen ($GABA_B$ antagonist).

Statistical evaluations of the effect of sTMS on the neuronal response to microiontophoretic ejection of L-glutamate were made using the average rate of firing in

Hz evoked during each epoch of microiontophoretic application. Five baseline pulses of L-glutamate were analysed to avoid variations of the responses of a cell between individual pulses and the reliability of the measurements was tested using Cronbach's alpha. The response of each cell under test conditions was examined as followed: (a) L-glutamate (baseline), (b) L-glutamate post-sTMS or (a) L-glutamate at the presence of bicuculine (baseline), (b) L-glutamate at the presence of bicuculline post-sTMS or (a) L-glutamate at the presence of saclofen (baseline), (b) L-glutamate at the presence of saclofen post-sTMS. Effects of sTMS or sham stimulation (two sTMS pulses of 0 V) were analysed using an ANOVA for repeated measures followed by Student's paired t-test, using the average of the baselines of all tested parameters for comparisons or compared with vehicle control using an independent samples t-test. When the assumption of sphericity with regard to the factor of repeats was violated, the Greenhouse–Geisser correction was applied. Data expressed as mean percentage of the baseline activity ± SEM.

Cortical spreading depression studies

Statistical analysis of the data was completed using SPSS Statistics 23 (IBM, USA) using non-parametric analysis, as data lacked normal distribution. Within group comparisons of electrical stimulation thresholds were analysed using the Wilcoxon signed-rank test. Effects of sTMS between groups were analysed using the Mann Whitney test. Significance was assessed at the $P < 0.05$ level. All data are expressed as the median value (μCoulombs) and interquartile range (IQ; 25% – 75% range).

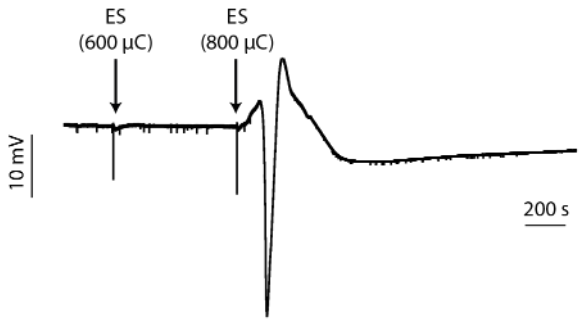

Figure 27: Representative example of an electrically evoked CSD wave in an animal treated with sham sTMS. Subthreshold stimulation with 600 μC; at 800 μC a wave of CSD is evoked

In vivo imaging:

LIM (.nd2) files were obtained from NIS Elements AR (Version 4.30.01, Nikon, Japan) and analysed using ImageJ Fiji (version 1.52d). GCaMP fluorescence was assessed using five randomly selected 497.18 x 497.18 µm regions of interest (ROI), avoiding blood vessels and unusually bright baseline fluorescence (caused by damaged tissue).

The mean fluorescence for each ROI for each frame of the video was extracted using the Multi Measure tool in the ROI manager and exported to Excel (Office 16, Microsoft Corporation, USA). The file was trimmed around the cortical event including a stable baseline. The raw fluorescence absolute signal values were normalised to allow for comparison between different experiments. A baseline period of stable fluoresce was recorded and averaged for each ROI, all subsequent changes in fluoresce were calculated as ΔF/F.

$$\Delta F/F = \frac{F_t - F_0}{F_0}$$

Where Ft is the fluorescence at time t and F0 is the fluorescence average over a baseline period (Chisholm et al., 2018).

Blood vessel diameters after the cortical events (sTMS and CSD) were normalised as a percentage of their baselines. Velocity of the CSD wave from the pinprick site to the ROI was recorded in addition to properties of the CSD wave: peak fluorescence, area under the curve, rise time, and decay time (Fig. 33C). Effects of sTMS or CSD were analysed using an rmANOVA for repeated measures with Bonferroni post hoc correction for multiple comparisons followed by Student's paired t-test. Data expressed as mean of the baseline recordings ± SEM. Properties of the CSD waves were analysed using an Independent Samples T-test for mean variance and two-sample Kolmogorov-Smirnov test for data distribution.

Results

sTMS inhibited spontaneous cortical activity

A total of 36 cortical neurons were recorded in 36 animals. Only neurons that demonstrated stable spontaneous activity under baseline conditions were selected for treatment with two consecutive sTMS pulses. Overall cells were located at an average depth of 798 ± 53 µm below the brain surface (Fig. 28A) and the average spontaneous neuronal firing rate was 4.41 spikes/sec ± 0.33.

sTMS applied at ~0.2 T (N = 6, P = 0.11), ~0.38 T (N = 6, P = 0.50), ~0.57 T (N = 6, P = 0.44) or 0.74T (N = 6, P = 0.14) had no significant effect on spontaneous neuronal firing. sTMS applied at ~0.92 T significantly reduced spontaneous neuronal firing ($F_{2.7,13.6}$ = 3.5; P = 0.047), by a maximum of 51.3% at 55 min (t_5 = 9.81; P < 0.001; Fig. 28C). The greatest reduction of spontaneous neuronal firing was seen after treatment with two pulses ~1.1T (N = 6; $F_{1.8, 7.4}$ = 5.4; P = 0.037), with maximum reduction of 66% occurring at 75 min post-sTMS (t_5 = 4.59; P = 0.01, Fig. 28 B,C). The maximum output used did not induce any motor responses.

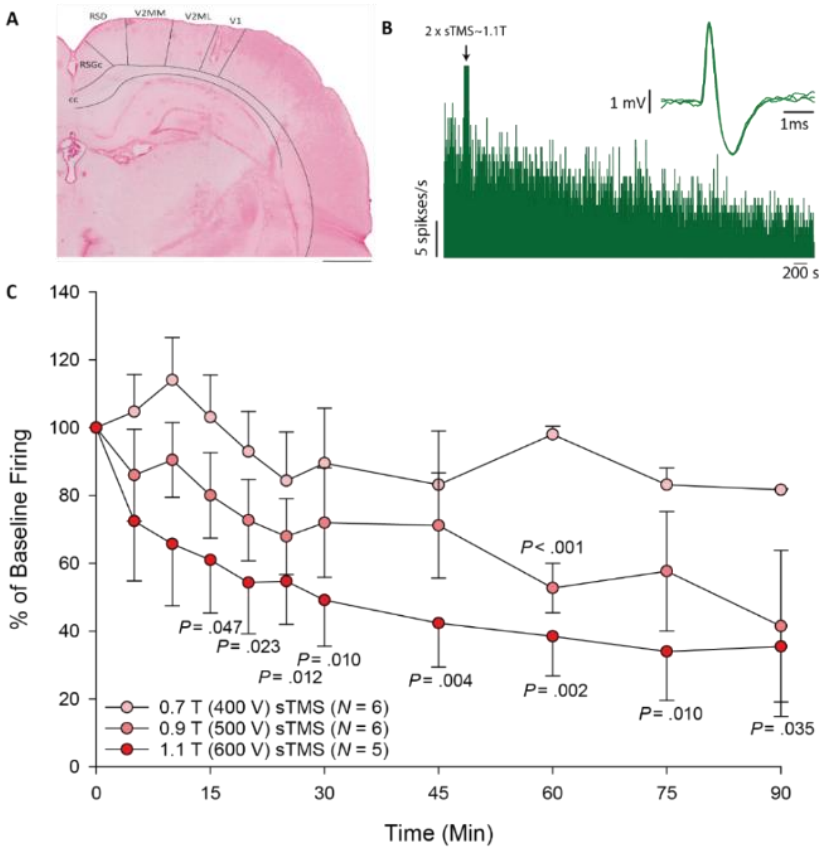

Figure 28: sTMS inhibits spontaneous cortical activity in a dose dependent manner *(A) Representative example of brain slice, showing electrode scar in V1 cortex (B) Representative example of reduction in cortical activity following 2x 1.1 T sTMS pulses. (B) 2x 0.7 T sTMS pulses had no significant effect on the spontaneous neuronal activity recorded of the occipital cortex (One way ANOVA,* $F(2.447, 12.237) = 2.232$, $p = 0.143$*), Overall 2x 0.9 T sTMS pulses had significantly reduced the spontaneous neuronal activity recorded of the occipital cortex (One way ANOVA,* $F(2.719, 13.595) = 3.541$, $p < 0.05$*), 2x 1.1 T sTMS pulses significantly reduced the spontaneous neuronal activity the spontaneous neuronal activity recorded of the occipital cortex (One way ANOVA,* $F(1.848, 7.393) = 5.4$, $p < 0.05$*)*

sTMS inhibited L-glutamate evoked-firing, but not in the presence of a GABA antagonist

A total of 27 (in 10 animals) and of 31 cortical neurons (in 7 animals) responding to L-glutamate microiontophoresis were recorded in the sham (2x 0 T) and sTMS (2x 1.1 T) groups, respectively. In the presence of bicuculline 12 cortical neurons (in 7 animals) were recorded in the sham (2x 0 T) and 11 cortical neurons (in 7 animals) in the sTMS (2x 1.1 T) group. In the presence of saclofen 18 cortical neurons (in 8 animals) were recorded in the sham (2x 0 T) and 12 cortical neurons (in 6 animals) in the sTMS (2x 1.1 T) group. Neurons were located at an approximate depth of 800 µm from the cortical surface (Fig. 29A).

The average glutamate induced neuronal firing rates were: 8.43 spikes/sec ± 13.28 in the sham group, 8.79 spikes/sec ± 13.16 in the sTMS group, 8.67 spikes/sec ± 13.07 in the sTMS with Bicuculline group and 10.41 spikes/second ± 12.08 in the sTMS with Saclofen group. The sTMS treatment of 2 pulses at 1.1 T was chosen based on outcomes from the dose-response studies on spontaneous neuronal activity presented above.

Between 0 T sham and 1.1 T sTMS groups, there was no difference across the mean firing of the five repeated epochs recorded during baseline (sham: $F_{4,16} = 0.98$, $P = 0.46$; sTMS: $F_{2,7} = 0.73$, $P = 0.92$) and all baseline responses were reliable (Cronbach's $\alpha \geq 0.88$). sTMS sham stimulation (2 x 0 T) had no effect on L-glutamate-evoked firing ($F_{2.9,2305} = 1.4$, $P = 0.24$; Fig. 29A, C). sTMS applied as two consecutive pulses at ~1.1 T significantly reduced L-glutamate evoked-firing, by 39.1% ($F_{3.9,7610} = 3.5$; $P = 0.014$; Fig. 29B, C).

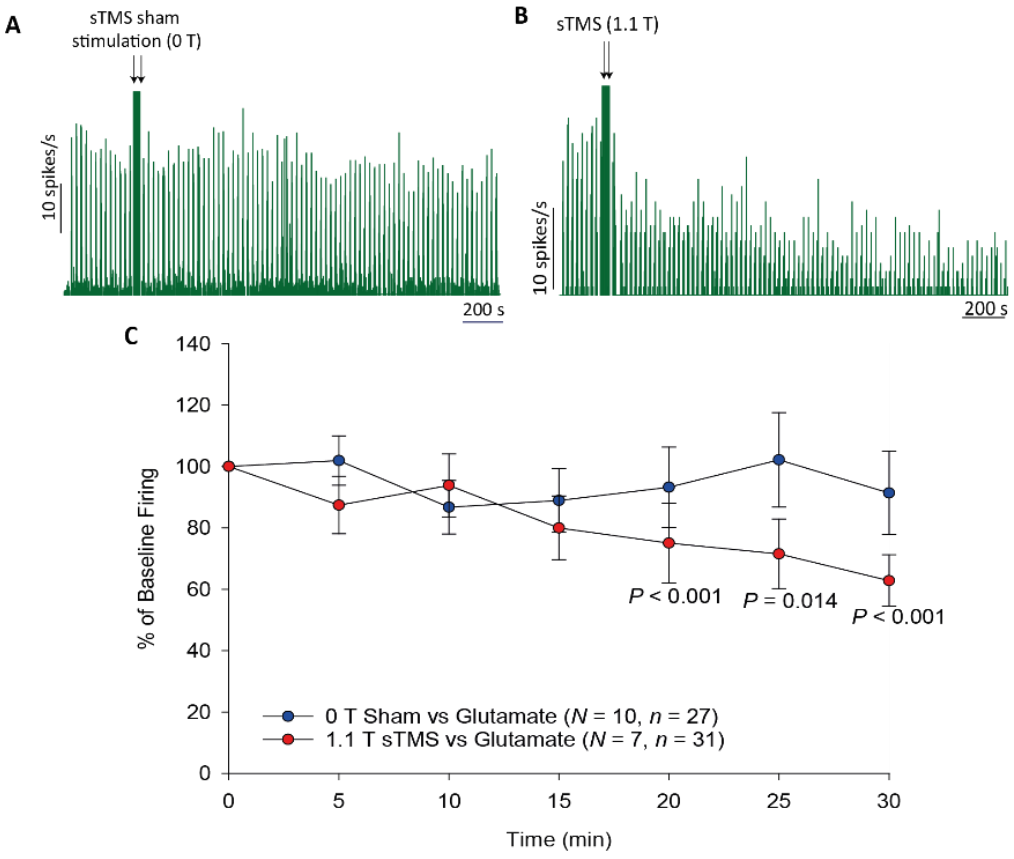

Figure 29: sTMS inhibits glutamate induced cortical activity *(A) Representative example of glutamate induced cortical activity with control (0 T) sTMS pulse (B) Representative example of reduction in glutamate induced cortical activity following two 1.1 T sTMS pulses (C) 2x 1.1 T sTMS pulses significantly reduced glutamate induced cortical activity (Independent Samples Test,* $t(377) = 2.467$, $p < 0.05$*)*

However, in the presence of GABA antagonists, two pulses of 1.1 T sTMS was not significantly different when compared to the 0 T sham group: Bicuculline ($GABA_A$ Antagonist) (t (188) = -1.541, P = 0.125, Fig. 30A C); Saclofen ($GABA_B$ Antagonist) (t (198) = 0.17, P = 0.865, Fig. 30B C). Two pulses of sTMS in the presence of a GABA antagonists were also significantly different than two pulses of sTMS alone: Bicuculline ($GABA_A$ Antagonist) (t (278) = -7.943, P < 0.001, Fig. 30D); Saclofen ($GABA_B$ Antagonist) (t (291) = -5.416, P < 0.001, Fig. 30D).

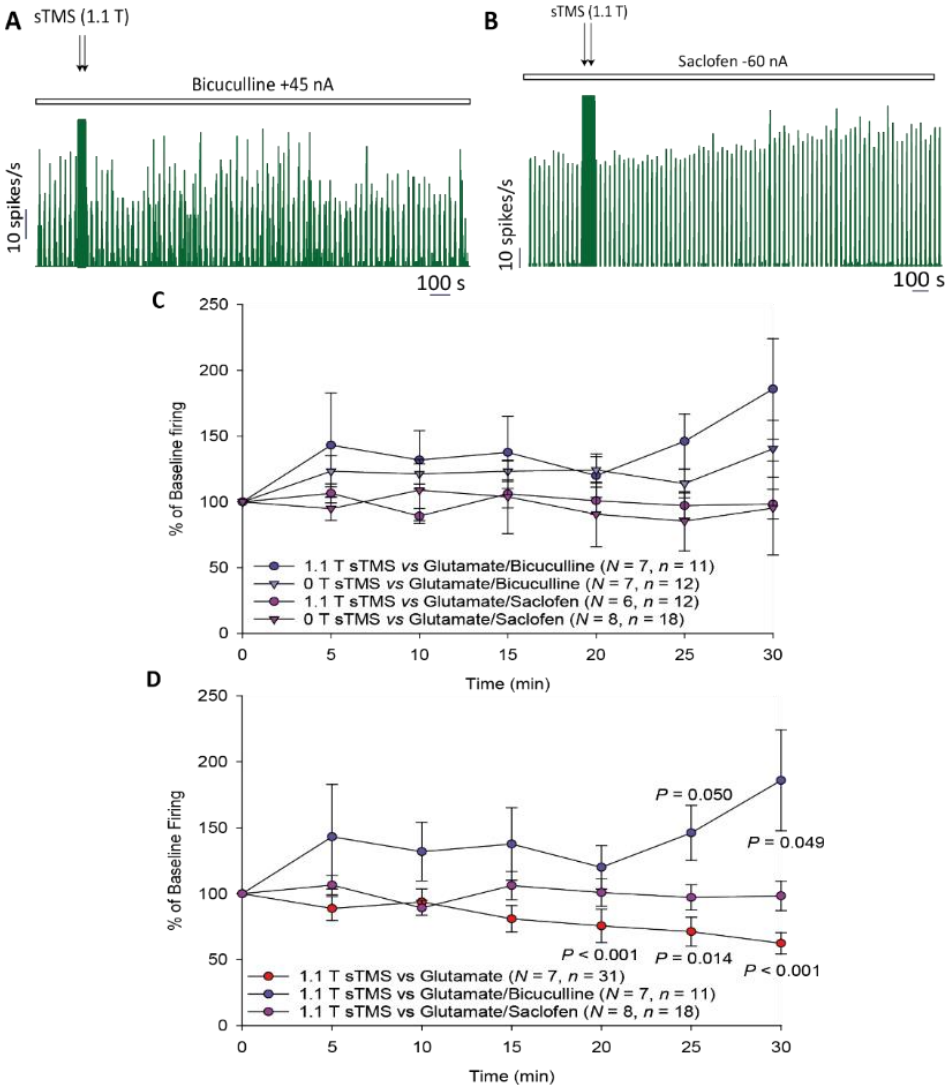

Figure 30 : sTMS inhibits glutamate induced cortical activity but not in the presence of GABA antagonists *(A) Representative example of glutamate induced cortical activity following two 1.1 T sTMS pulses in the presence of $GABA_A$ antagonist bicuculline (B) Representative example of reduction in glutamate induced cortical activity following two 1.1 T sTMS pulses in the presence of $GABA_B$ antagonist saclofen (C) 2x 1.1 T pulses had no significant effect on glutamate induced cortical activity compared with 0T controls in the presence of GABA antagonists, Bicuculline (Independent samples T-test, p = 0.125) and Saclofen (independent samples t-test, p = 0.865) (D) Comparison of glutamate responses to 2x 1.1 T sTMS with glutamate alone, with bicuculline and with saclofen*

sTMS elevated electrical stimulation threshold for cortical spreading depression, but not in the presence of a GABA antagonist

We determined the electrical stimulation threshold for CSD (Fig. 31A) in the sham-treated group (N = 8; median: 1200, IQ:500-1600 µC; Fig. 31 B) and in the active sTMS treated group (N = 8; median: 1600, IQ:1600 - 3200 µC). In the active sTMS treatment (2x 1.1 T) this threshold was significantly raised (P = 0.16, r = -0.85; Fig. 31B).

However, in the presence of GABA antagonists two pulses of sTMS at the same intensity electrical stimulation threshold for cortical spreading depression was not significantly increased. Both bicuculline (N = 5; U = 3.0, P = 0.40, r = -0.23; Fig. 31B) and saclofen (N = 5; U = 16.50, P = 0.599, r = -0.146; Fig. 31B) remained the same as 0 T sham control group while being significantly different than the electrical CSD threshold with sTMS alone (bicuculline: N = 13; U = 3.0, P = 0.011, r = -0.71; saclofen: N = 13; U = 1.50, P = 0.006, r = -0.77).

In a different experimental group, the CSD threshold was determined at baseline conditions (median:1200, IQ:600 - 1600 µC). Following two consecutive sTMS pulses at ~1.1 T, induction of CSD was attempted every 30 minutes for 2 hours until a CSD was induced. Only one animal produced CSD using the same cortical stimulating parameters 30 min after sTMS. In the remaining animals at 2 h post-sTMS, parameters for cortical stimulation were increased until a CSD wave was elicited (median: 2400, IQ:1400 - 3200). Overall, sTMS treatment significantly increased by two-fold the CSD induction threshold (N = 8; P = 0.017, r = -0.84; Fig. 31C).

sTMS had no effect on cortical GCaMP fluorescence

To assess if the sTMS parameters used in electrophysiological experiments (600 V, ~1.1 T) to inhibit cortical activity and CSD were actually exciting cortical neurons acutely, sTMS at 1.1 T was applied over the visual cortex of Snap25-2A-GCaMP6s-D mice while GCaMP fluorescence was recorded. sTMS had no effect on GCaMP fluorescence (P = 0.134; Fig. 32A, B & C). As a positive control, needle prick-induced CSD at a later time point significantly changed GCaMP florescence ($F_{3.2,156.8}$ = 18.5; P < 0.05; Fig. 32A & C).

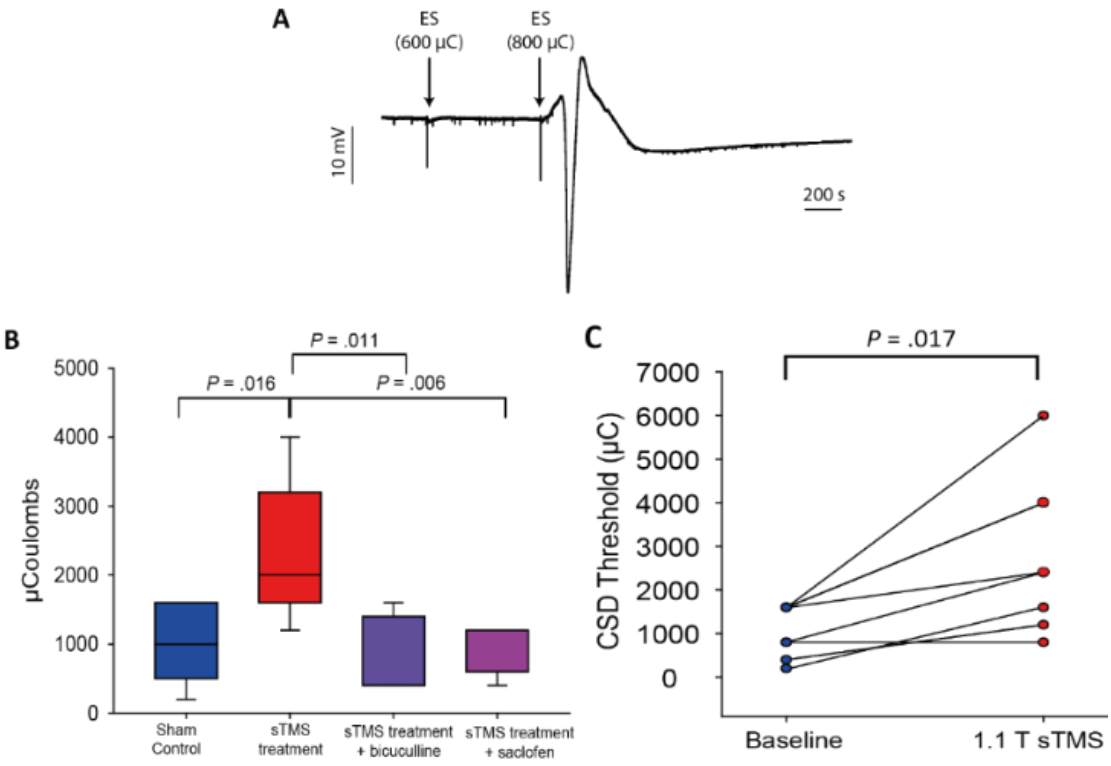

Figure 31: sTMS increases threshold for electrically induced CSD but not in the presence of GABA antagonists *(A) Representative example of CSD waves induced by electrical stimulation showing subthreshold electrical stimulation (600 µC) and CSD threshold electrical stimulation (800 µC) (B) sTMS at ~1.1 T significantly increased the electrical threshold required to generate a CSD compared to the sham stimulation group (0T). (P = 0.016; Mann-Whitney test), there was no significant difference in the presence of GABA antagonists bicuculline (P = 0.011; Mann-Whitney test) and saclofen (P = 0.006; Mann-Whitney test) (C) Two sTMS pulses at ~1.1 T significantly increased the electrical threshold required to generate a CSD, compared to the pre-sTMS baseline threshold (P < 0.05; Wilcoxon test).*

sTMS had no effect on cortical blood vessel diameter

To assess the potential actions of sTMS on superficial blood vessels, the fluorescent dye dextran was injected in C57BL/6 mice. sTMS at 1.1 T was applied over the visual cortex of mice while Dextran fluorescence was recorded. The diameter of blood vessels was measured pre- and post-sTMS. sTMS had no effect on Dextran fluorescence ($P \geq 0.82$; Fig. 32A B D). As a positive control, needle prick-induced CSD significantly increased the diameter of blood vessels ($F_{1.2, 6.1} = 7.2$; $P < 0.05$; Fig. 32A & D).

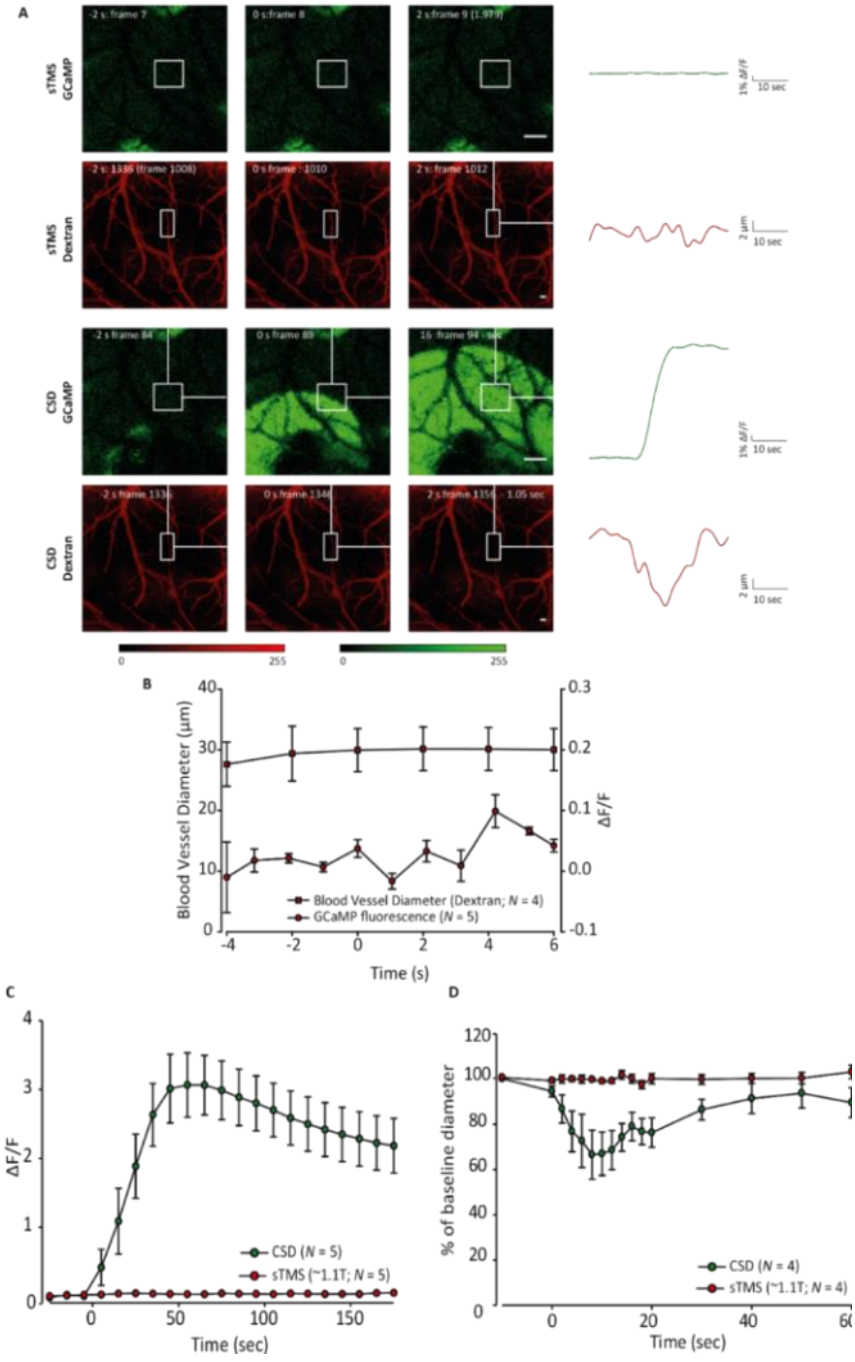

Figure 32: sTMS does not directly stimulate cortical neurons *(A) Examples of cortical imaging immediately before, during and after cortical event of either 2x 1.1 T sTMS pulse or pinprick induced CSD. In vivo calcium imaging in Snap25-2A-GCaMP6s-D mice was used to visualise the cortical calcium uptake as a marker for cortical activity. Cortical blood vessels were visualised via Dextran Tetramethylrhodamine, 70,000 MW, Lysine Fixable. (B) 2x 1.1 T sTMS pulses have no immediate effect of cortical GCaMP fluorescence (One way ANOVA, F(1.41, 26.784) = 2.762, p = 0.097) or cortical blood vessel diameter (One way ANOVA, F(1.241, 6.095) = 0.139, p = 0.777) (C) Pinprick induced CSD significantly increases GCaMP fluorescence in the cortex but 2x 1.1 T sTMS has no effect (Paired T-Text, t(415) = 20.999, p <0.05, r = 0.718) (D) Pinprick induced CSD significantly decreases cortical blood vessel diameter in the cortex but 2x 1.1 T sTMS has no effect (Paired T-Text, t(90) = -9.36, p <0.05, r = 0.702)*

sTMS had a significant effect on cortical spreading depression parameters

To assess the potential actions of sTMS on CSD once initiated following treatment with active sTMS stimulation, GCaMP fluorescence was recorded in Snap25-2A-GCaMP6s-D mice. sTMS significantly reduced the peak fluorescence (t (58) = 2.968, P = 0.004, Fig. 33D) and area under the curve (t (58) = 3.189, P = 0.002, Fig. 33G) of the CSD wave compared to sham treatment. In addition, the resolution from peak back to baseline was also significantly reduced (t (58) = 5.292, P < 0.001, Fig. 33H). However, the rise time to peak was not significantly different (t (57) = 0.888, P = 0.378, Fig. 33E). The mean velocity of the CSD wave was not significantly different between 0 T sham and 1.1 T sTMS groups (t (57) = 0.465, P = 644), however, the distribution was significantly different (D (59) = 0.385, P = 0.025, Fig. 33F).

When the pinprick was repeated after 30 minutes the mean peak GCaMP fluorescence from the resulting CSD wave did not change from baseline (2.172 ± 0.384 ΔF/F) when no sTMS was applied (2.36 ± 0.591 ΔF/F) (Fig. 34A D). However, when 2x 1.1 T sTMS was applied between the first and second induced CSD waves the mean fluorescent peak dropped (1.245 ± 0.303 ΔF/F)[2] (Fig. 34A D). In animals receiving 2x 1.1 T sTMS application prior to the first CSD, the mean peak fluorescence was not significantly different at 10 minutes (1.009 ± 0.119 ΔF/F) and at 40 minutes (1.004 ± 0.093 ΔF/F) post-stimulation (t (60) = -0.155, p = 0.231, Fig. 34B D). Additionally, CSD were more difficult to induce post-sTMS than in the naïve group, pre-sTMS CSD's were induced on 6/6 (100 %) of attempts, whereas post-sTMS 15/29 (51.7 %) successfully induced CSD's (Fig. 34F).

[2] Unfortunately, since the treatment group (N = 6) was split in half to show the change within animals, the resulting N numbers are too small to apply meaningful statistical testing.

Chapter 2:

Acute direct effects of sTMS application on Cortical Neuronal Activity

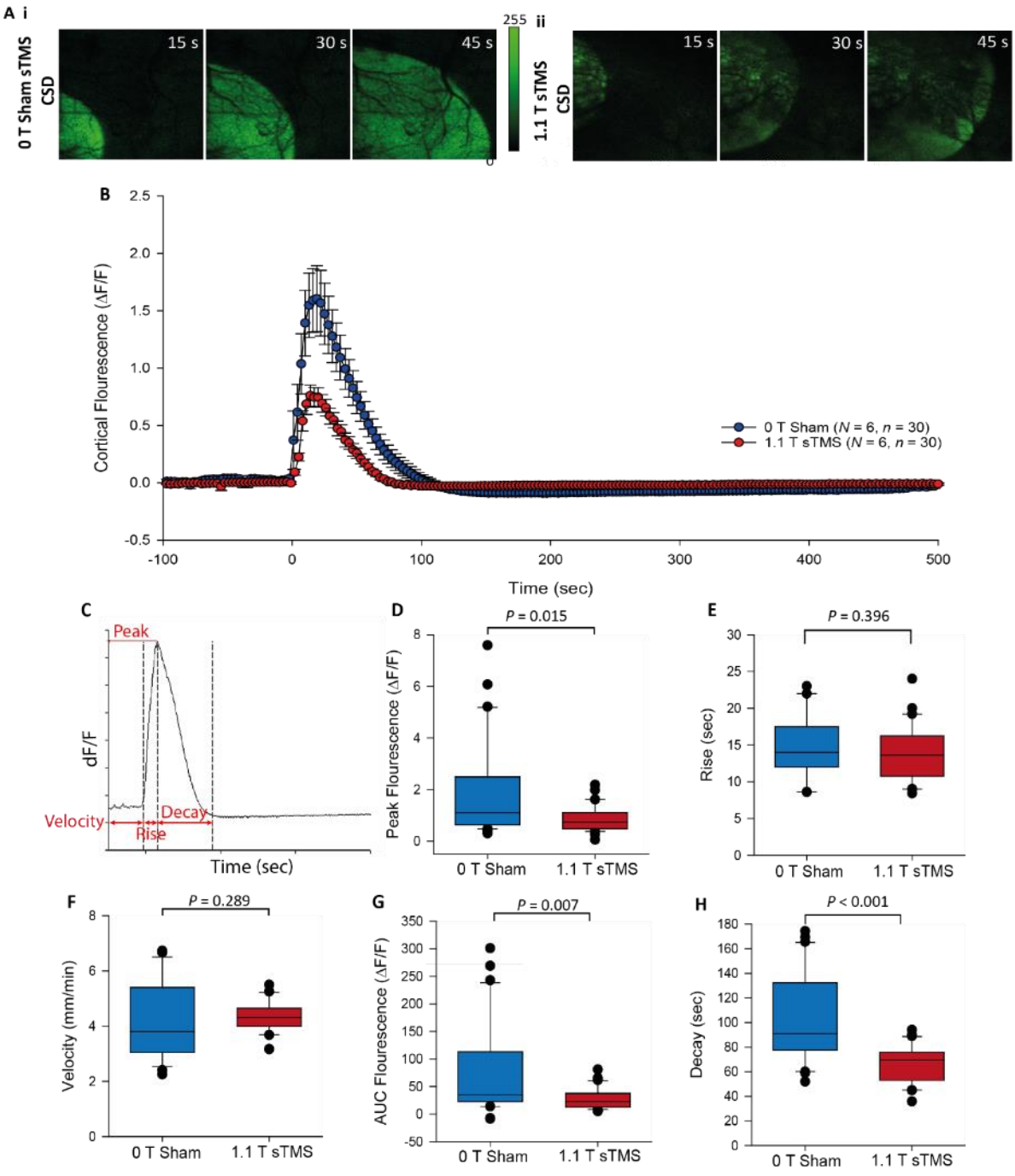

Figure 33: sTMS had a significant effect on cortical spreading depression parameters *(A) examples of cortical in vivo imaging during a cortical spreading depression i) in naïve animals ii) in animals pre-treated with 2x 1.1 T sTMS (B) Mean cortical fluorescence during a pinprick induced CSD wave of naïve and sTMS pre-treated animals (C) Diagram illustrating parameters of the cortical spreading depression wave measured (D) 2x 1.1 T sTMS significantly decreased peak fluorescence (P = 0.015; Mann-Whitney test) (E) 2x 1.1 T sTMS had no significant effect on the rise time to peak (P = 0.396; Mann-Whitney) (F) 2x 1.1 T sTMS had no significant effect on the velocity of the CSD wave (P = 0.289; Mann-Whitney test) (G) 2x 1.1 T sTMS significantly decreased the area under the curve of cortical fluorescence during a CSD wave (P = 0.007; Mann-Whitney test) (H) 2x 1.1 T sTMS significantly decreased the decay time from peak back to baseline (P < 0.001)*

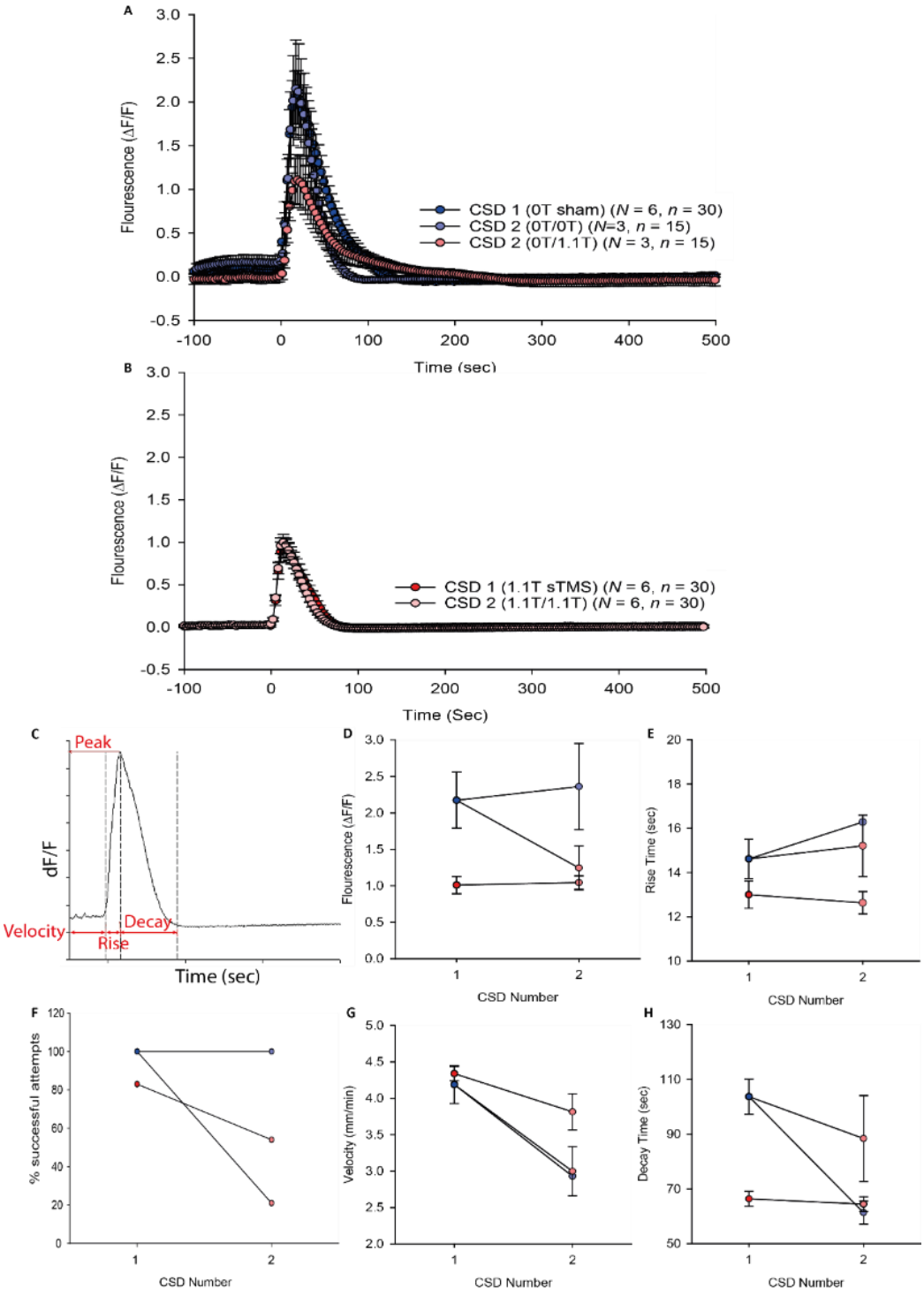

Figure 34: sTMS had a significant effect on cortical spreading depression parameters *(A) The mean peak GCaMP fluorescence from the resulting CSD wave did not change from baseline (2.172 ± 0.384 ΔF/F) when no sTMS was applied (2.36 ± 0.591 ΔF/F). A reduction in mean fluorescent peak (1.245 ± 0.303 ΔF/F) occurred when 2x 1.1T sTMS was applied between the first and second induced CSD (B) The mean peak fluorescence was not significantly different at 10 minutes (1.009 ± 0.119 ΔF/F) and at 40 minutes (1.004 ± 0.093 ΔF/F) following 2x 1.1T sTMS application (t (60) = -0.155, p = 0.231) (C) Diagram illustrating parameters of the cortical spreading depression wave measured (D-H) effect of 2x 1.1T sTMS on parameters of multiple pinprick-induced CSD waves*

Discussion

The primary purpose of this study was to identify the acute actions of single pulse transcranial magnetic stimulation (sTMS) application on neuronal activity of the cortex. The data presented suggest that sTMS at these parameters, does not excite cortical neurons nor influence cortical vascular tone. However, sTMS can reduce spontaneous neuronal activity in the visual cortex, potentially by engaging inhibitory GABAergic activity rather than directly supressing glutamatergic excitatory activity. These mechanisms could explain our findings that sTMS increased the threshold of induction of cortical spreading depression (CSD), given that sTMS had no effect in this model in the presence of a GABA antagonists. The data do not exclude the possibility that alternative sTMS protocols would produce better results, but they do offer a significant advancement of our knowledge on the cortical mechanism of action of sTMS in migraine treatment and demonstrate an electrophysiological cortical mechanism of action that involves the GABAergic system.

We found that sTMS can induce a dose-dependent inhibition of cortical spontaneous activity with the most effective stimulations being between 0.9 - 1.1 T, which is very close to the clinically used sTMS intensity. To explore whether this inhibitory effect was achieved by supressing excitatory cortical activity, we excited cortical neurons by microiontophoresis of glutamate and then applied the sTMS treatment. We found that indeed sTMS at ~1.1T inhibits excitatory glutamatergic activity. However, it remained unclear if this occurs by direct influence on glutamatergic excitation or by engaging inhibitory connections that depress neuronal firing. To investigate this, the GABA receptor competitive antagonists, bicuculline and 2-hydroxy saclofen, were continuously applied onto single neurons during pulsed excitation with L-glutamate. In this experiment, sTMS had no effect in blocking glutamatergic excitation, suggesting its effects are indirect by influencing the GABAergic system.

Andreou et al., (2010) have previously shown that one pulse of sTMS with a rise time of 170 μs was more effective in blocking mechanically or chemically induced CSD compared to lower rise time pulses. However, the actual acute actions of sTMS on cortical activity have not previously been investigated. Here, we employed a much smaller coil of 11 mm diameter, which allowed a more focal stimulation, compared to the size of coil

used in other TMS studies in rodents, which varied between 5 - 7 cm. The smaller coil offered the advantage of positioning it nearer the recording site. We also used two consecutive pulses of sTMS as a treatment dose, given that clinically patients will also use at least two pulses as an acute treatment.

The sTMS-induced reduction of spontaneous and glutamatergic cortical activity found in our study may explain the sTMS actions on blocking CSD. Our data suggest that this is achieved by increasing the threshold of CSD induction. The electrically evoked CSD model used in this study has the advantage of establishing the stimulation threshold needed to excite cortical neurons in order to induce a CSD wave, additionally, the stimulus can be repeatedly applied without damaging the cortex. We show that indeed pre-treatment with sTMS increases the electrical threshold required to induce a CSD. We also show that the sTMS treatment can block for at least 2 hours the induction of a CSD when using the baseline threshold electrical stimulation. However, in the presence of GABA antagonists, sTMS has no effect in this CSD migraine model.

This would concur with the work of Murphy *et al.* (2016) who suggested that sTMS activates inhibitory $GABA_B$ receptors in the upper cortical layers which in turn inhibit activity of dendritic pyramidal neurons in layer V of the somatosensory cortex. By blocking $GABA_B$ receptors they were able to prevent inhibitory effects of sTMS on the somatosensory cortex (Murphy et al., 2016). At the depth of around 800 um we recorded from, we presume that recordings were similarly made from large pyramidal neurons in lamina V (Gabbott and Stewart, 1987), as interneurons, of various types, appear to be in more superficial lamina (Fröhlich, 2016). However, although every effort was made to record from neurons that displayed similar action potential characteristics, and as close to a cell soma as possible (indicated by the biphasic shape of the action potential), the nature of such *in vivo* recordings does not allow us to be 100% certain of the type of neurons we recorded from. These outcomes further confirm a mechanism of action for sTMS that involves GABAergic neurotransmission. Of interest, GABA agonists were previously shown to inhibit CSD in migraine models.

CSD itself is characterized as a slow wave of depolarisation of neurons and glial activation in the cortex, followed by a short-lasting depression. This phenomenon is accompanied by blood flow changes. Here, we employed *in vivo* confocal microscopy to

visualize cortical blood vessels in dextran-injected mice, during and post-sTMS application. sTMS had no immediate or short-term effect on the vascular tone, suggesting that its efficacy in blocking CSD is purely by interactions with cortical cellular activity.

A major disadvantage of the electrophysiological experiments performed here was the long duration (typically several seconds) of the stimulus artefact produced by the sTMS device during capacitor discharge. Single biphasic pulses to the cat visual cortex have previously been shown to elicit facilitation of activity in the first 500 ms followed by distinct inhibition lasting several seconds (or the inverse at stronger stimuli) (Moliadze et al., 2003). The artefact prevented the observation of immediate (<1 second) modulatory effects of sTMS on neuronal activity. To address this question, we employed in vivo calcium imaging and applied sTMS over the cortex of GCaMP-expressing mice. GCaMP is a genetically encoded calcium indicator which increases its fluorescence intensity with the uptake of calcium, thereby providing a measure of neuronal activation. In these experiments, sTMS had no immediate or short-term effect on GCaMP fluorescent signal, suggesting that at these stimulating parameters sTMS does not excite cortical neurons on average, at least in the superficial cortical layers. Given that sTMS does not excite cortical neurons, its influence on GABAergic activity found in this study, could potentially be through molecular changes at the $GABA_A$ receptor at least, and not by activation of GABAergic neurons. A study using voltage-sensitive dyes in cats found that sTMS at a higher intensity, applied at the visual cortex induced a brief focal activation, immediately followed by synchronous suppression of a large pool of neurons.

Limitations

There are several limitations to be addressed with these experiments. In this set of experiments, and indeed all following electrophysiological experiments in subsequent chapters, a dilute solution of 60 mg/kg pentobarbital sodium was used to anaesthetise the animals. This anaesthetic choice was used as it has been previously shown that pentobarbital effects CSD initiation and propagation equivalent to the lowest concentrations of volatile anaesthetics (Kitahara et al., 2001, Kudo et al., 2013, Kudo et al., 2008). In addition pentobarbital was used to be consistent with previous research (Andreou et al., 2016). Pentobarbital sodium is a short acting barbiturate used as an

anaesthetic and sedative, acting primarily as an agonist of $GABA_A$ receptors to enhance inhibitory GABA action (Suckow et al., 2012). As we've shown that sTMS appears to have interactions with GABAergic mechanisms, the use of pentobarbital could limit the effect of sTMS on cortical mechanisms seen. This could be improved with the use of non-GABAergic anaesthetics, for example ketamine, however these have shown to have a greater effect on cortical functioning. Ideally the acute effects of sTMS on the cortex should be evaluated on non-anaesthetised awake animals, this could be achieved with an implanted microelectrode array recording from awake animals (Baker et al., 1994). However, as this would require an additional level of ethics approval, such experiments are not feasible in our department.

Due to the nature of the electrophysiological studies being performed here treatment blinding was not possible. However, the experimental protocols were designed to minimise the possibility of experimenter influence. The recording parameters and thresholds were set prior to the start of recording and were not altered during the experiment or analysis, thus allowing confidence in group comparisons. Additionally, when more than one treatment was used, randomization was applied, i.e., when applying sTMS pulses at different voltages or GABA antagonists. The cortical neuronal response to sTMS has previously been suggested to be state-dependent (Silvanto et al., 2007, Pasley et al., 2009). Activity of the neurons in the cortex pre-stimulus could affect the response to sTMS, if the neurons are inhibited prior to the stimulus, the result is facilitation of the activity of the neuron, even if the sTMS paradigm was inhibitory (e.g. Low frequency rTMS) (Silvanto and Cattaneo, 2017). This was not controlled for during this set of experiments with all recorded cells pooled together. Future experiments could spike sort recorded activity into individual cells and threshold cellular activity based on firing rate. Any state-dependent actions of sTMS may also have ramifications for the glutamate-induced activity experiments. However, studies investigating state-dependency in TMS have been focused on the rTMS paradigm, it is unclear if this is also the case with sTMS.

Conclusion

In conclusion, this study, demonstrated that subthreshold application of sTMS can acutely inhibit neuronal activity in the cortex. This inhibition is potentially mediated via GABAergic inhibition by cortical interneurons. Reduced cortical excitation could provide a possible mechanism for how sTMS is able to increase the electrical threshold required to produce cortical spreading depression and why the peak neuronal activity during a CSD wave is significantly smaller after sTMS.

Chapter 3: Acute indirect effects of sTMS application on Thalamic and Hypothalamic Neuronal Activity

Chapter 3:

Acute indirect effects of sTMS application on Thalamic and Hypothalamic Neuronal Activity

Introduction

The hypothalamus is a small structure below the thalamus at the base of the brain. It has an important role in the control of homeostasis via neuroendocrine connections to the pituitary gland. Particularly it plays an important role for several autonomic functions including circadian rhythms and the stress response. The hypothalamus is highly interconnected with many areas of the central nervous system (CNS), with projections to many brain structures including; the cortex, the limbic system, the brainstem and the spinal cord (Settle, 2000).

As has been noted, migraine prevalence is sexually dimorphic, with 3x higher rates in females than males. This difference is believed to be due to fluctuating hormonal levels through the oestrous cycle in females. Migraine attack prevalence peaks at days -2 to +3 of the cycle and are at their lowest during oestrogens peak at around day 10-11 (MacGregor and Hackshaw, 2004), suggesting that decreased levels of oestrogen may provoke an attack. The hypothalamus regulates oestrogen production via a negative feedback loop. The hypothalamus causes the anterior pituitary to release follicle-stimulating hormone (FSH) which stimulates the granulose cells to produce oestrogen. It remains unclear if the driving factor in migraine is hypothalamic regulation of oestrogen, or oestrogenic feedback to the hypothalamus. The most frequently cited self-reported trigger for migraine is stress (Robbins, 1994), the hypothalamus regulates the stress response via the hypothalamic-pituitary-adrenal (HPA) axis (Settle, 2000), further implicating as having a role as a migraine trigger.

Many of the symptoms arising during the premonitory phase of migraine, including; sleep disturbance, mood changes, food craving, thirst and urination, are associated with hypothalamic activation (Giffin et al., 2003, Quintela et al., 2006). Yawning is one of the main predictive premonitory symptoms (Giffin et al., 2003) and is strongly associated with the neurotransmitter dopamine. Application of dopamine receptor agonists can provoke yawning in animal models (Mogilnicka and Klimek, 1977) via the paraventricular nucleus of the hypothalamus (Melis et al., 1987). This suggests that hypothalamic dopamine could play an important role in premonitory symptoms of migraine. The A11 dopaminergic nucleus of the hypothalamus has been shown to project

to the spinal cord and to offer the sole source of dopamine in the spinal cord. Stimulation of the A11 nucleus was shown to inhibit second order nociceptive neuronal activity (Fleetwood-Walker et al., 1988) in the spinal cord and the trigeminocervical complex TCC (Charbit et al., 2011).

Further evidence for hypothalamic modulation of the spinal cord has been shown through electrical stimulation of the medial preoptic area of the hypothalamus, which was shown to inhibit dorsal horn neurons of the lumbar (Carstens et al., 1982) and thoracic spinal cord (Lumb, 1990). The hypothalamus is also the sole producer of the neuropeptides orexin and somatostatin, both of which have been shown to modulate activity of the TNC (Bartsch et al., 2004b, Bartsch et al., 2005). Overall, the hypothalamus plays an important role in the descending modulation of trigeminal second order neurons.

Direct evidence for hypothalamic activation during the premonitory phase of migraine has come from brain imaging studies. Scanning within 4 hours of a spontaneous migraine attack onset, the hypothalamus was shown to be activated (Fig. 35) (in addition to brain stem and cortical activation shown previously (Weiller et al., 1995)), this hypothalamic activation persisted beyond pain relief with sumatriptan (Denuelle et al., 2007) suggesting that hypothalamic activation was not a consequence of pain, but rather a causal factor. Due to pixel resolution limitations, it was not possible to confirm which sub-nuclei within the hypothalamus became activated. That said, Danuelle *et al.* do suggest that the region of hypothalamic activation is more anterior than the area previously described for TAC's (Matharu et al., 2006, Matharu et al., 2004b).

Infusion of the migraine trigger nitric oxide donor glycerol trinitrate (GTN) has also been shown to induce premonitory symptoms (Afridi et al., 2004), and cause activation of the hypothalamus (as well as the brain stem and cortex) (Maniyar et al., 2013) equivalent to that seen in spontaneous attacks (Karsan et al., 2017).

Hypothalamic activation is also seen during attacks of other headache disorders including cluster headache (May et al., 1998), hemicrania continua (Matharu et al., 2004a) and paroxysmal hemicrania (Matharu et al., 2006).

Additionally, the hypothalamus can modulate spinal nociceptive processing indirectly, through connections to brain stem structures involved in the modulation of pain processing, including the periaqueductal grey (PAG) (Rizvi et al., 1996), raphe magnus (Murphy et al., 1999) and parasympathetic system through the superior salivatory nucleus (Hosoya et al., 1983). The hypothalamus has also been shown to have projections to the posterior and lateral posterior thalamus, that relays ascending trigeminothalamic neuronal signals (Kagan et al., 2013).

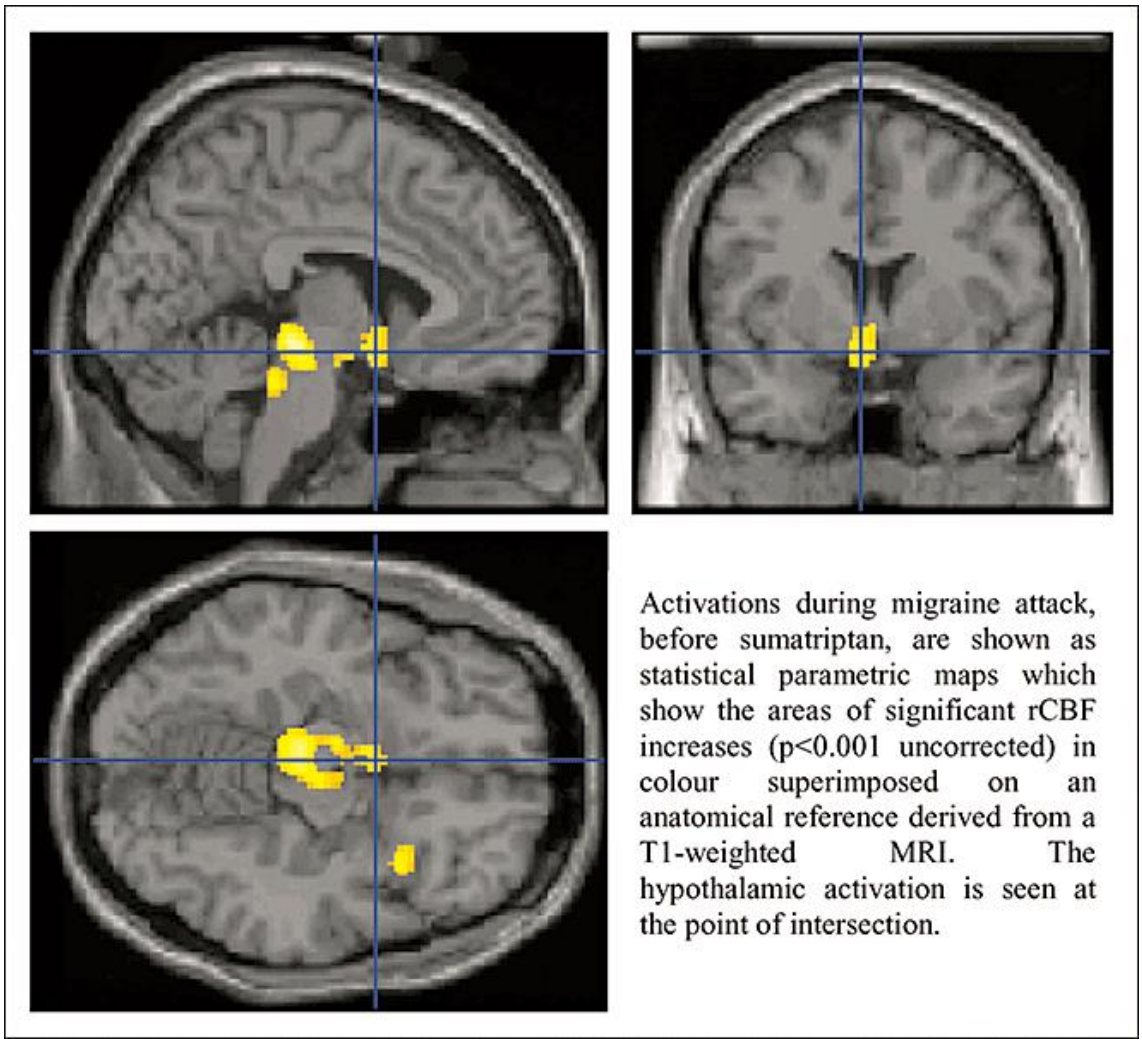

Figure 35: Hypothalamic activation during spontaneous attacks in 7 patients (Denuelle et al. 2007)

Chapter 3:

Acute indirect effects of sTMS application on Thalamic and Hypothalamic Neuronal Activity

The thalamus is an important relay centre of the ascending trigeminothalamic pathway, collating ascending trigeminal nociceptive signals, and disseminating those signals onwards to higher cortical areas (Fig. 36). The ventroposteromedial (VPM) thalamus, is the principal relay nucleus in migraine, relaying information from second order neurons of the TCC to cortical areas involved in the "pain-matrix", particularly the S1, S2 and insula cortices (Zagami and Lambert, 1990, Zagami and Lambert, 1991). The posterior, lateral posterior and lateral dorsal thalamic nuclei are also receiving inputs from the ascending trigeminothalamic pathway. Third order neurons in these nuclei become activated following stimulation of dural trigeminal fibres and have ascending projections to the S1, S2 parietal association, retrospinal, auditory, ectorhinal, motor and visual cortices (Fig. 36) (Noseda et al., 2011).

Thalamic neurones demonstrate two distinct firing patterns, tonic and burst firing. during normal sensory processing the thalamic cell demonstrates a tonic firing pattern. When hyperpolarised for 100 ms or more thalamic neurones demonstrate a characteristic high frequency burst of action potentials that lasts for approximately 100 ms. Burst firing is dependent on Low voltage-activated (LVA) T-type calcium channels (Dreyfus et al., 2010, Destexhe et al., 1998). There are three subtypes of t-type calcium channels; $Ca_V3.1$, $Ca_V3.2$, and $Ca_V3.3$. T-type channels are inactive at membrane potentials of –50 to –60 mV but become primed when the neurone is hyperpolarised (- 100 mV) for 100 ms or longer. When hyperpolarised, for example by inhibitory input, low voltages can open the cell allowing calcium into the cell but is inactivated at a fast rate (100 ms) resulting in a high frequency but transient burst of action potentials (Cain and Snutch, 2010).

Thalamic burst firing is especially important in slow wave sleep (Anderson et al., 2005) when theta bursts of firing synchronise large populations of cells, however, has also been shown in awake animals (Ramcharan et al., 2000) where burst activity could be important for attention. Alteration in thalamic burst firing has been suggested to play a role in neuropathic pain states (Lenz et al., 1989) however it is unclear what role burst firing may play in migraine.

Figure 36: Relative laminar density of terminal arbors of dura-sensitive thalamocortical neurons. *Fibre density (0 – 4) is colour coded according to the scale at the bottom (Noseda et al., 2011)*

Comparing PET scanning of migraine patients between inter-ictal periods and spontaneous migraine attacks has shown thalamic activation (in addition to activation of cerebellum, insula and cingulate, prefrontal and temporal cortices) (Fig. 37) (Afridi et al., 2005a). The migraine attack is characterised not only by severe pain but also by a number of other associated symptoms, including sensory sensitivities; photophobia, phonophobia and osmophobia. One proposed pathway for photophobia was found using single unit recordings and tract tracing in animal models. Cells from non-imaging forming photosensitive retinal ganglion cells were found to project along the optic nerve to the posterior thalamus. Additionally, the thalamus has further projections to the visual cortex which may contribute further to the development of photosensitivity (Noseda et al., 2010). A similar pathway has been found in human subjects using diffusion MR tractography (Maleki et al., 2012b). Additionally, an association between phonophobia and cutaneous allodynia (Ashkenazi et al., 2010) would also support the role of the thalamus in sensory sensitivity.

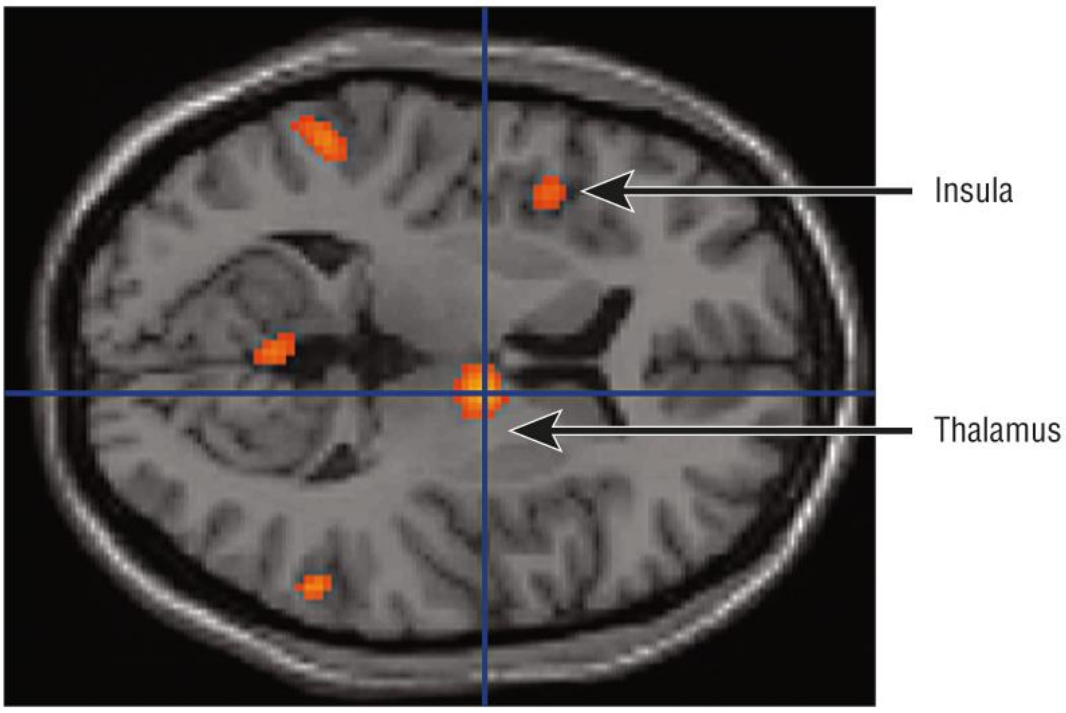

Figure 37: Activation of the thalamus and insula in the migraine state (Afridi, Giffin et al. 2005)

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Centrally acting preventative migraine treatments such as propranolol (Shields and Goadsby, 2004), sodium valproate (Andreou et al., 2010a), and small molecule CGRP receptor agonists (Summ et al., 2010) have been shown to modulate thalamic activity. Suggesting the thalamus is an effective target for preventative treatments for migraine.

Interaction between the thalamus and the cortex is not a unidirectional process, there is a dynamic feedback loop back and forth. The cortex provides a great deal of input to the thalamus, with 10x as many descending corticothalamic axons as ascending thalamocortical projections (Deschênes et al., 1998).

Corticothalamic axons originate in lamina VI of the cortex and provide direct excitatory input to thalamic cells, they additionally project to the thalamic reticular formation (TRF), a separate structure surrounding the main thalamus that provides inhibitory GABAergic projections to the thalamus (Kim et al., 1997, Cox et al., 1997). Low frequency corticothalamic firing (<0.1 Hz), causes an initial thalamic discharge via the direct monosynaptic pathway which is then followed by a larger and longer-lasting inhibition via the indirect thalamic reticular formation pathway. High frequency cortical firing (~10 Hz) caused facilitation of the excitatory monosynaptic input and a depression of the inhibitory pathway, resulting in an overall increase of thalamic activity (Crandall et al., 2015). Initial excitation from low frequency corticothalamic firing activates AMPA glutamate receptors, depolarising the thalamic neuron. High frequency corticothalamic firing removes the magnesium plug from the NMDA glutamate receptor further depolarising thalamic neurones (MacDermott and Dale, 1987). Simultaneously, high frequency corticothalamic firing caused depression of the inhibitory input from the TRF, thought to be due to short-term depression of the GABAergic TRF synapse (Crandall et al., 2015).

Relevant to migraine, cortical depression (induced by K+) produced persistent depression of cortical synaptic activity, enabled production of hyperpolarisation-activated cation currents (burst firing) in thalamic dorsal lateral geniculate neurons (Nita et al., 2003).

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The current generated by single-pulse transcranial magnetic stimulation (sTMS) is localised within the cortex directly beneath the coil, therefore, is considered a purely cortical treatment. However, several studies have shown that rTMS and/or sTMS has an indirect action on limbic structures it could not directly stimulate; the thalamus, hypothalamus, hippocampus, putamen, pulvinar and insular (Li et al., 2004, Andreou et al., 2016, George et al., 1999, Gur et al., 2004). Application of 1 Hz repeated TMS (rTMS) was shown to be able to increase activity the mediodorsal nucleus of the thalamus in depressed patients (Li et al., 2004). In animal models, sTMS' was shown to modulate the third order neuron activity in the VPM thalamic nucleus, blocking both spontaneous neuronal activity and activity arising from ascending c-fibre stimulation (Andreou et al., 2016). This effect is believed to be driven by activation of cortico-thalamic pathways by the sTMS pulse. Application of rTMS to the prefrontal cortex, has been shown to modulate activity of the hypothalamus (George et al., 1999, Gur et al., 2004). Long term rTMS application was found to reduce glutamate and GABA levels in the hypothalamus, while increasing levels in the hippocampus and striatum in animal models (Yue et al., 2009). To date there have been no studies focusing on sTMS' action on the hypothalamus.

In this chapter the acute effects of the migraine sTMS treatment will be investigated in two structures; the VPM thalamus and the hypothalamus. The VPM in the thalamus relays trigeminal nociceptive signals from spinal second order neurons onwards to the cortical "pain matrix" along third order neurons. The extent of thalamic activity which is relayed onto the cortical "pain-matrix" is dependent on the excitability of the thalamic neurons, and therefore is subject to modulation. Previous preliminary studies have shown that cortical events, such as CSD, can influence thalamic activity (Andreou et al., 2012). The hypothalamus containing descending projections, which modulate spinal dorsal horn activity (Skagerberg et al., 1982). Electrical stimulation of the hypothalamus inhibits trigeminally evoked activity of the TCC, and conversely lesioning of the hypothalamus facilitated stimulus evoked activity of the TCC (Charbit et al., 2009).

I will use electrophysiology to investigate if how cortically applied sTMS affects neuronal VPN thalamic and hypothalamic activity. Additionally, I will investigate how CSD

affects the activity of these sub-cortical areas, and whether this changes following cortical application of sTMS.

To record from these deeper brain structures stereotaxic electrophysiology will be used. This involves using coordinates from a rat brain atlas, along with the electrode and holder calibrated against a standardised interaural point, to place the tip of the recording electrode in the approximate location within the brain[3]. A secondary method is required to confirm the electrode is in the correct location. The third order thalamic neurons being targeted, receive input from second order neurons, thus the trigeminal periorbital receptive field can be used as confirmation of the recording location during the experiment. The hypothalamic neurons are not activated by peripheral input and thus have no receptive field that can be used. Instead, following the termination of the experiment dye will be injected into the recording location, allowing for later examination of the tissue. A tyrosine hydroxylase stain was used to mark dopaminergic cells in the A11 hypothalamic nucleus as a histological landmark to compare the recording location from.

The focus of this chapter is to investigate the effects of sTMS on brain structures relevant to migraine (thalamus and hypothalamus) that are not directly stimulated by the magnetic pulse (and subsequent electrical current). The main hypothesis is that sTMS could influence these migraine related structures indirectly via activation of cortico-thalamic and cortico-hypothalamic pathways.

[3] So long as the animal is within a weight range (for rats: 250 – 350 g)

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Methods

Animals and ethical approvals

All experiments were performed in accordance with the United Kingdom Home Office Animals (Scientific Procedures) Act (1986). Experiments were carried out in adult male Sprague Dawley rats (*N* = 102, 250 - 350 g; Charles River, UK). Rats were housed on a 12/12-hour light/dark cycle with food and water available *ad libitum*. All experiments were performed under general anaesthesia and terminated by anaesthesia overdose and/or cervical dislocation.

Surgery

Rats

General anaesthesia was induced with intraperitoneal injection of 60 $mg.kg^{-1}$ pentobarbital sodium (Merial, UK). Supplementary anaesthesia was maintained with continuous intravenous infusion of pentobarbital (12-15 $mg.kg^{-1}.h^{-1}$). A tracheotomy was performed to permit ventilation of the animal and end-tidal expired CO2 was monitored and maintained between 3.5 - 4.5% (Capstar-100, CWE). The left femoral vein and artery were cannulated to allow for constant intravenous infusion of anaesthetic and monitoring of blood pressure, respectively. Adequate anaesthesia was gauged by the absence of toe pinch withdrawal and eye-blink reflexes and gross changes in blood pressure. Core temperature was monitored and maintained near 37°C using a homoeothermic blanket system (TC-1000, CWE). Animals were fixed on a non-magnetic stereotaxic frame (Kopf instruments). Craniotomies were performed and dura mater incised to expose the occipital and parietal cortex for electrophysiological recordings/stimulations.

Craniotomy for stereotaxic recordings

For recording in deeper brain nuclei stereotaxic placement of the recording electrode was used. The electrode was lined up on the locating pin of a Calibrated A/P Zeroing Bar (1450, Kopf Instruments, USA) and the anterior/posterior (A/P), medial/lateral (M/L) and dorsal/ventral (D/V) coordinates noted. Coordinates for the nuclei were taken from "The

Rat Brain in Stereotaxic Coordinates" (Paxinos and Watson, 2006). Coordinates for the hypothalamus were: A/P: 5.5, M/L: 0.5 and D/V: 2.2 - 1.8 from interaural. The coordinates for the VPM thalamic nucleus were: A/P: 5.2, M/L: 3.0, and D/V: 4.5 - 5.0 from bregma (electrode placement confirmed via brush stimulation of the V1 dermatome) (Fig. 38). Correct placement of the rat onto the stereotaxic frame was particularly important during stereotaxic recording to ensure accurate electrode placement. The ear bars were adjusted to an even depth and the nose bar moved, so the head was level and straight. Additionally, an alignment tool (944, Kopf Instruments, USA) was used to confirm correct placement on the stereotaxic frame. Once on the stereotaxic frame, the skull was drilled, as previously described, in the approximate location on the right hemisphere of the skull.

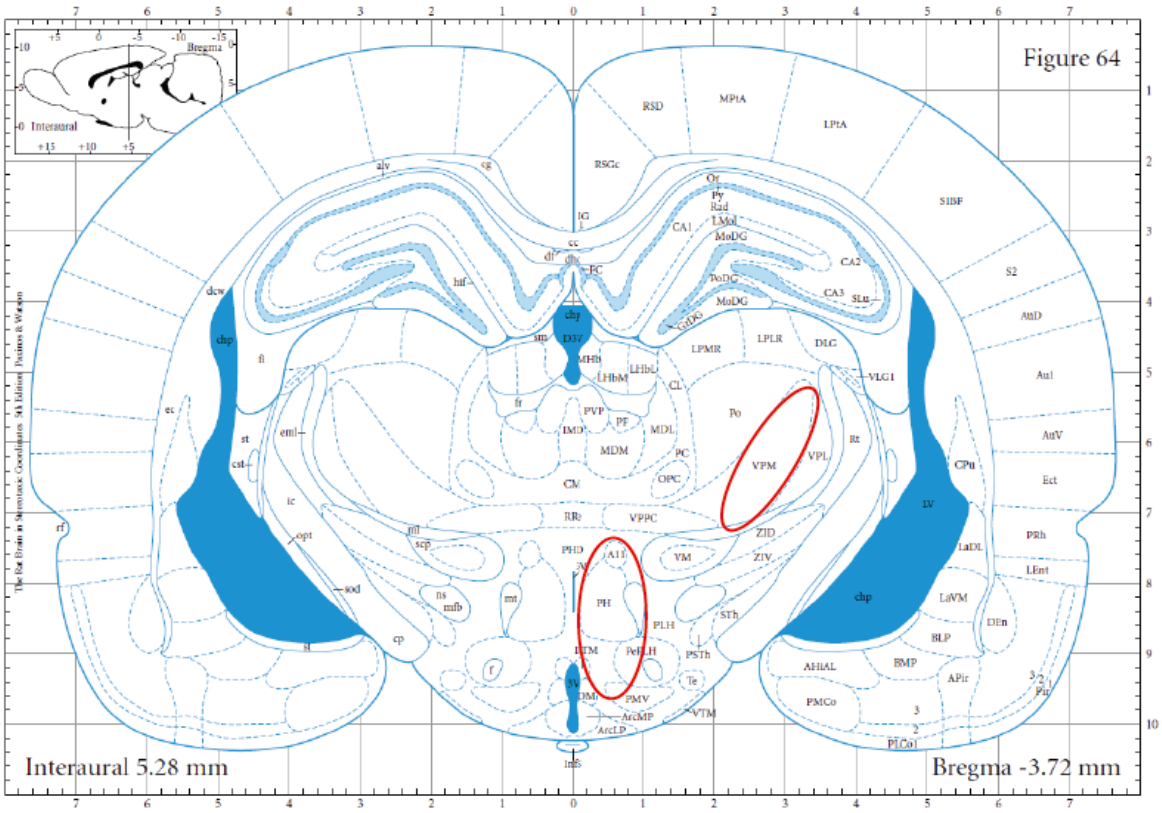

Figure 38: Stereotaxic location of VPM thalamic nucleus and hypothalamic target, indicated in red circles (Paxinos and Watson, 2006).

sTMS effect on the electrical activation threshold of third order VPM thalamic neurons

A cranial window was additionally drilled over the approximate location of the VPM also exposing the superior sagittal sinus (SSS). The trigeminal primary afferent fibres innervating the SSS were stimulated by lowering a pair of platinum wire stimulating electrodes onto the SSS using an electrode manipulator (1460, Kopf Instruments, USA). Care was made to only make contact with the sinus and not the cortex (Fig. 39A). Evoked potentials were recorded from single third order neurons and stored online Spike2, as previously described. Evoked potentials were collected as a post-stimulus trace that mimics a storage oscilloscope. The voltage of the stimulus was increased between 5 - 40 V until a consistent response in thalamic third order neurons was seen, while the duration of the pulse (0.1 ms) was kept constant. Once the electrical activation threshold was found, this was confirmed by reducing the stimulus to the previous sub-threshold stimulation to confirm that the action potentials, which represent an ALL or NOTHING response, were not seen, before returning to threshold stimulation. Thalamic stimulation thresholds were found in 15 separate cells per animal in both 0 T sham and 1.1 T sTMS group (Fig. 39B).

Effects of sTMS between groups were analysed using the Mann Whitney test. All data are expressed as the mean threshold value (volts) ± SEM and significance assessed at the $P < 0.05$ level (SPSS version 23.0; SPSS Inc., USA).

CSD effects on third order VPN neurons, and actions of sTMS

Baseline spontaneous activity of third order neurons, in the VPM thalamic nucleus, was recorded for at least 15 min before a pinprick to the occipital cortex was used to induce a CSD. Two pulses of 1.1 T sTMS or 0 T sTMS (sham group) were applied to the occipital cortex 1 min pre-CSD induction by pinprick. Neuronal activity was recorded for 90 minutes post-CSD. For each unit, firing rate in spikes.sec-1 was averaged in 5 minute intervals for the first 30 min, and at 15 min intervals afterwards. At least 15 minutes of stable baseline activity pre-stimulus was averaged to give a baseline. Post-CSD thalamic

activity was compared against this baseline (Fig. 39C). As responses to CSD were variable, thalamic spontaneous response to CSD was grouped into; facilitated and inhibited if it differed more than ± 2X standard deviation (SD), or unaffected if any change from baseline was < 2X SD. A single CSD was induced per animal, to prevent damage to the cortex.

In addition to spontaneous activity, trigeminovascular evoked activity of third order neurons was assessed, using stimulation of the trigeminal fibres innervating the SSS. Fifteen electrical pulses of 30 V, 0.1 ms at 0.5 Hz, were applied to the SSS using a pair of platinum wire stimulating electrodes and evoked responses were collected as cumulative post-stimulus histograms (PSH). Frequency and latency of thalamic activity following stimulation was recorded, allowing identification of Aδ-fibre (latency: 6 – 15 ms) and C-fibre (latency: >16 ms) induced activity. These PSH were taken every 5 minutes for 15 minutes prior to stimulation and averaged for a stable baseline. sTMS and CSD were applied as above. PSH were taken at 1, 5, 10, 15, 20, 25, 30, 45, 60, 75 and 90 minutes post-CSD (Fig. 39C). Pre-stimulus PSH were averaged to give a baseline. Post-CSD PSH's were compared against this baseline. Subsequent analysis of the thalamic response to trigeminal stimulation following CSD were grouped based on the spontaneous activity response (facilitates, inhibited or unaffected).

Effects of sTMS on spontaneous and evoked thalamic response to CSD were analysed using the Mann Whitney test. Repeated measures analysis of variance (rmANOVA) was computed with two factors: sTMS and Repeats, to determine sTMS evoked thalamic response to CSD. When the assumption of sphericity with regards to the factor of Repeats was violated, adjustments were made for the degrees of freedom and P values according to the Greenhouse-Geisser correction. All data were expressed as a mean percentage of baseline ± SEM and significance assessed at the $P < 0.05$ level (SPSS version 23.0; SPSS Inc., USA).

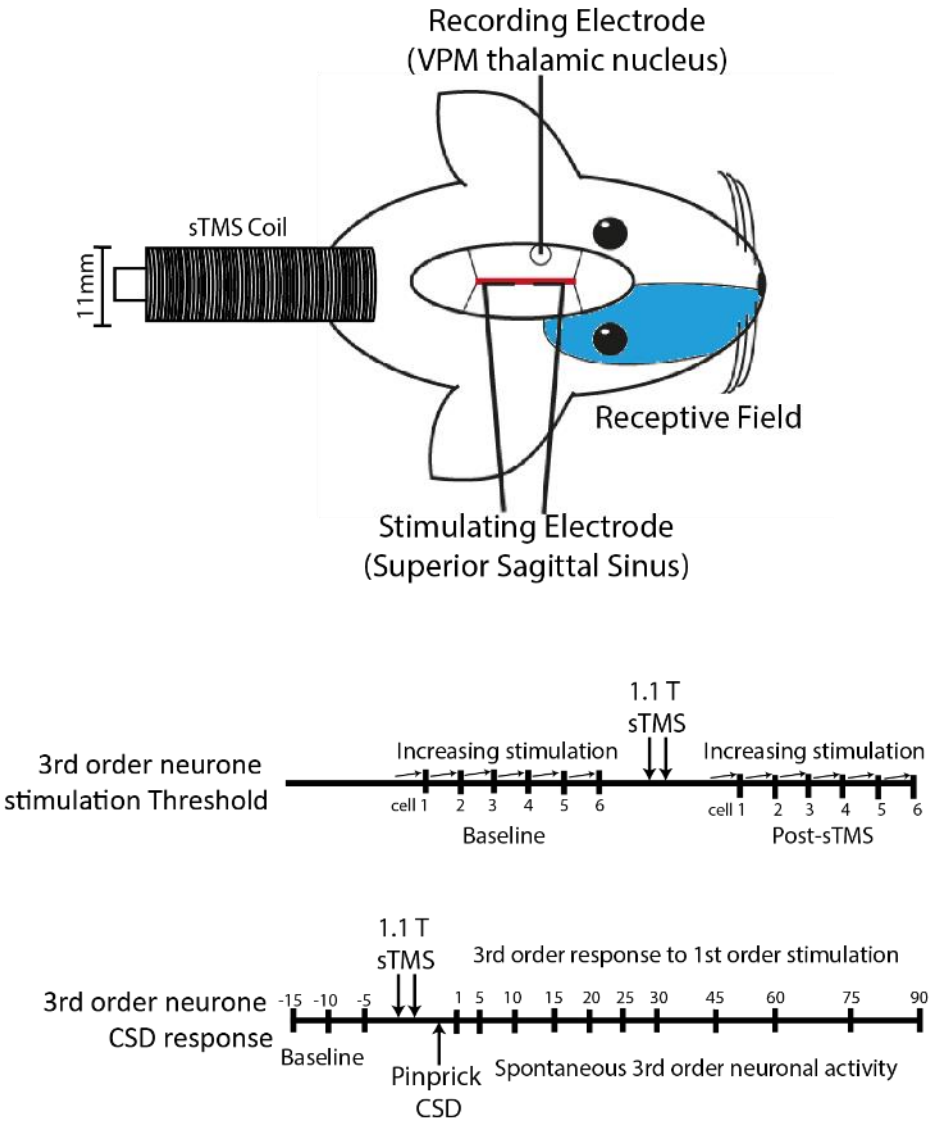

Figure 39: sTMS indirect effect on Ventroposteromedial thalamus *(A) Experimental setup in the rat model showing sTMS application site, and positions of stimulating and recording electrodes (B) Diagram illustrating the experimental protocol of indirect effect of sTMS on Ventroposteromedial thalamic thresholds for trigeminovascular stimulation (C) Diagram illustrating the experimental protocol of indirect effect of sTMS on Ventroposteromedial thalamic activity following CSD, PSH were taken at 1, 5, 10, 15, 20, 25, 30, 45, 60, 75 and 90 minutes post-CSD*

sTMS' effect on hypothalamic spontaneous activity

Extracellular spontaneous activity from single units in the hypothalamus was recorded using a carbostar-7s recording/iontophoresis combination microelectrode (E1073, Kation Scientific LLC, USA) with an impedance of ~1 M (Fig. 40A). Upon identifying

spontaneously firing cells, baseline activity of at least 15 min was recorded. A CSD was induced via mechanical stimulus and hypothalamic spontaneous neuronal activity recorded for 30 minutes post-CSD, to confirm corticohypothalamic connection.

Secondarily effect of single sTMS pulse to hypothalamic activity was investigated. Following a 10 minute baseline, a single sTMS pulse was applied to the cortex and hypothalamic activity recorded for up to 10 minutes post-stimulus. sTMS stimulations were repeated a further 3 times every 10 min (Fig. 40B).

Post-hoc spike sorting was carried out to isolate biphasic, dopaminergic neurons using Spike2. For CSD response, the firing rate (in spikes.sec-1) was averaged for each unit in 1 min intervals for the first 5 minutes post-CSD and subsequently every 5 minutes up to 30 minutes. For sTMS response the firing rate (in spikes.sec-1) was averaged for each unit in 1 min intervals for 10 minutes post-CSD.

Effects of sTMS on hypothalamic response to CSD and sTMS were analysed using the Mann Whitney test. All data were expressed as a mean percentage of baseline ± SEM and significance assessed at the $P < 0.05$ level (SPSS version 23.0; SPSS Inc., USA).

Hypothalamic Histology

Upon completion, animals were terminated, and perfused via intracardiac perfusion using a 60 ml/min pump (313S, Watson Marlow, UK). Animals were exsanguinated with heparinised (MEHEP14, Wockhardt, UK) 0.9 % saline (S9888, Sigma-Aldrich, USA) and 4% paraformaldehyde (16005R, Sigma-Aldrich, USA) Trigeminocervical complex and brains were removed and stored in 30 % sucrose solution with 0.05 M sodium azide (S2002, Sigma-Aldrich, USA). Preserved tissues were frozen in OCT compound (361303E, VWR Chemicals, Belgium) and sectioned into 30 µm slices using a UV cryostat (CM1860, Leica, Germany) and placed onto Superfrost microslides slides (3610108, VWR Chemicals, Belgium). Brains were sectioned in the coronal plane and trigeminocervical complex sectioned in the transverse plane. Slides were dried overnight and stored at 4ºc.

After two 5 minute washes in TBS plus 0.025% Triton X-100, sections were incubated for 2 h in a blocking solution of 5% Normal Goat Serum (Vector, S-1000). Twenty-four-hour primary incubation with Rabbit polyclonal Anti-Tyrosine Hydroxylase antibody primary antibody (ab112, Abcam) 1:500 dilution using a diluent solution made up with diluted in TBS with 1% BSA at 4°C. After primary incubation and Rinse 2x 5 min TBS 0.025% Triton, sections were incubated for 60 minutes in Goat Anti-Rabbit IgG H&L (Alexa Fluor 488) (ab150077, Abcam) at 1:250 dilution.

After a wash in water, the sections were mounted on slides, air dried, dehydrated through graded alcohol solutions and cover slipped with DPX medium (Fisher Scientific). Representative brightfield images were taken using an Axio Imager.Z1 microscope with a 10x lens (Zeiss, Germany).

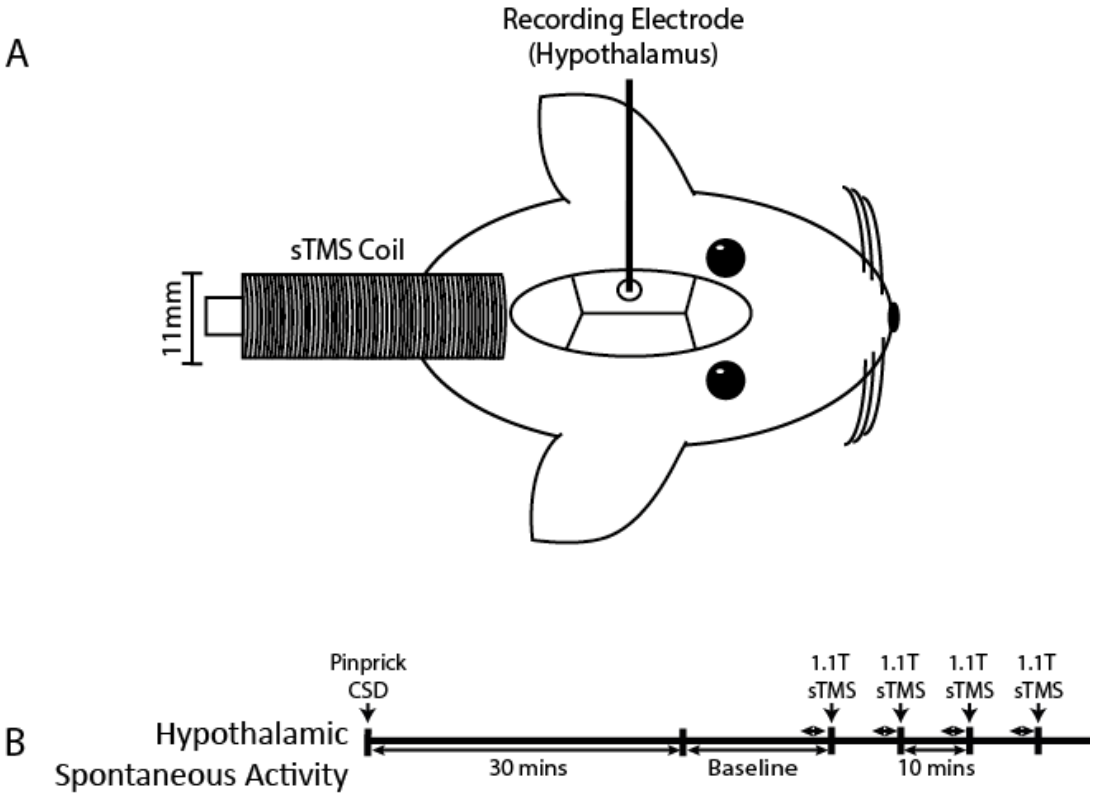

Figure 40: Acute sTMS indirect effect on hypothalamic activity electrode *(A) Experimental setup in the rat model showing sTMS application site, and positions of recording (B) Diagram illustrating the experimental protocol of indirect effect of sTMS on hypothalamic spontaneous neuronal activity*

Results

sTMS increases activation thresholds of thalamic third order neurons

The electrical stimulation threshold for third order neurons of the VPM thalamic nucleus was determined in naïve animals (N = 6); with an average threshold of 18.25 ± 1.93 V. Following 2x 1.1 T sTMS application the average threshold was 25 ± 3.16 V (Fig. 41A). Following sTMS treatment the activation threshold of 3rd order thalamic neurons was significantly raised (U = 354, P = 0.001, r = -0.393; Fig. 41B).

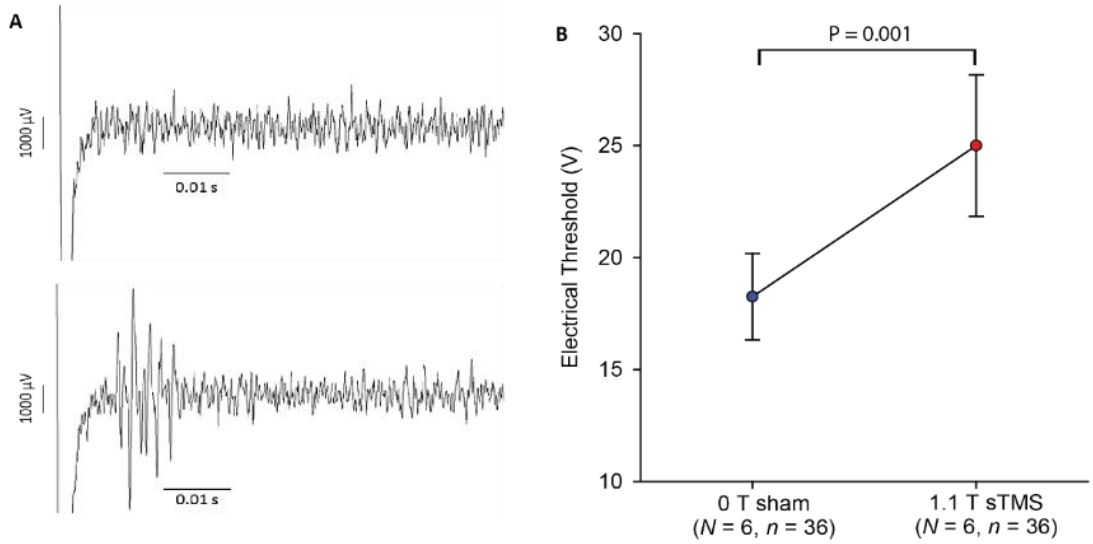

Figure 41: sTMS increases activation thresholds of thalamic third order neurons *(A) Representative example of thalamic activation driven by peripheral trigeminal stimulus (B) application of 1.1 T sTMS significantly increased the threshold electrical stimulus required to produce thalamic activity (P = 0.001; Mann-Whitney)*

CSD modulates thalamic neuronal activity

Previous research showed that in naïve animals inducing a CSD by pinprick modulates activity of trigeminothalamic neurons (Andreou et al., 2012). In naïve control animals, 35% (N = 26) of the spontaneous neuronal activity, recorded from third order thalamic cells, was inhibited from pre-CSD baseline with an average firing of 84.63% ± 3.26 of the baseline. In 39% (N = 29) of recorded cells spontaneous activity of thalamic third order neurons were facilitated from pre-CSD baseline, with an average firing rate of 114.05% of baseline ± 8.56. There was no effect on spontaneous neuronal activity in 26%

of trigeminothalamic neurons (*N* = 19), with average firing rate 98.15% of pre-CSD baseline ± 1.80 (Fig. 42A).

The thalamic response to stimulation of trigeminal first order neurons of the superior sagittal sinus was found. Neuronal activity occurring 0.006 – 0.015 seconds after a stimulus was categorised as evoked from fast myelinated Aδ-fibres. Activity with a latency of > 0.016 seconds after a stimulus were categorised as slower unmyelinated C-fibres (Fig. 42A). The inhibited group (*N* = 26) had an average Aδ-fibre induced firing rate of 87.21% ± 2.53 of baseline firing and an average firing rate of 86.00% ± 2.86 of C-fibre induced activity. The facilitated group (*N* = 29) had an average Aδ-fibre induced firing rate of 110.33% ± 2.47 of baseline firing and an average firing rate of 153.43% ± 9.64 of C-fibre induced activity. The unaffected group (*N* = 19) had an average Aδ-fibre induced firing rate of 99.64% ± 1.20 of baseline firing and an average firing rate of 93.85% ± 2.23 of C-fibre induced activity (Fig. 42B & C).

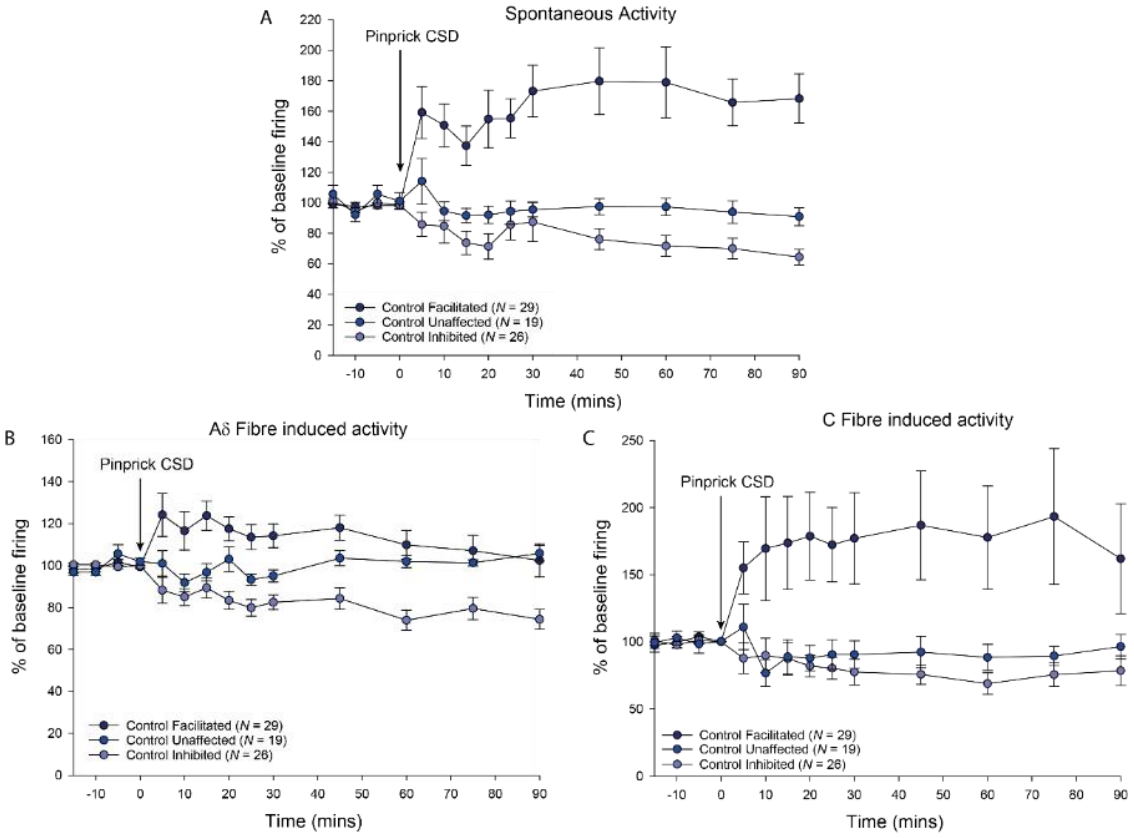

Figure 42: CSD modulates thalamic neuronal activity in naïve animals *(A) CSD modulates spontaneous activity of third order thalamic neurons (B) CSD modulates Aδ-fibre induced thalamic activity (C) CSD modulates C-fibre induced thalamic activity*

sTMS' affects thalamic spontaneous neuronal activity in response to CSD

Following application of 2x 1.1T sTMS pulses to the occipital cortex, 80% (N = 8) of trigeminothalamic neurons demonstrated inhibited spontaneous neuronal activity, with an average reduction of 26.52% ± 9.54 of baseline firing (Fig.43A). There was a significant difference seen in the extent of thalamic inhibition between naïve and sTMS pre-treated animal groups post-CSD (N = 14; U = 53, P = 0.04; Fig. 43B). Only two trigeminothalamic recordings were found to be facilitated following CSD with an average 94.32% ± 21.29 increase of baseline firing, but no further analysis was carried out due to low N number.

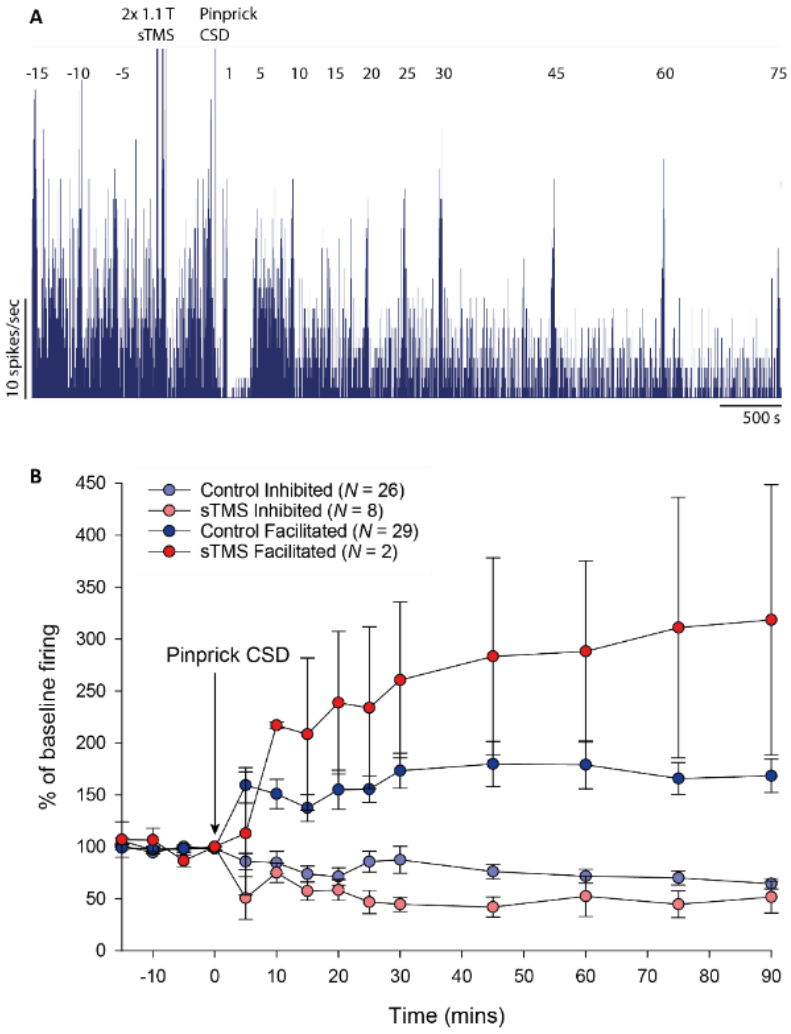

Figure 43: sTMS' affects thalamic spontaneous neuronal activity response to CSD *(A) representative example of spontaneous thalamic activity following a pinprick induced CSD (B) Both control and sTMS pre-treated thalamic activity is inhibited by pinprick induced CSD, the extent of inhibition was significantly different between sTMS and control groups (P = 0.04, Mann-Whitney)*

In sTMS pre-treated animals, with inhibited spontaneous thalamic activity following CSD (N = 8) the average Aδ-fibre induced firing rate of 97.98% ± 2.11 of baseline firing. CSD had no significant change in Aδ-fibre evoked firing in pre-treated animals ($F_{2.4,16.7}$ = 6.2; P = 0.576). This was significantly different than the control animals (N = 14; U = 45, P = 0.014; Fig. 44B).

The C-fibre induced activity, in the same group had an average of 100.90% ± 6.4 of baseline firing. CSD had no significant change in C-fibre evoked firing in pre-treated animals ($F_{1.7,10.9}$ = 0.91; P = 0.444). This was significantly different than the C-fibre induced activity in the inhibited group of control animals (N = 14; U = 49, P = 0.024; Fig. 44C).

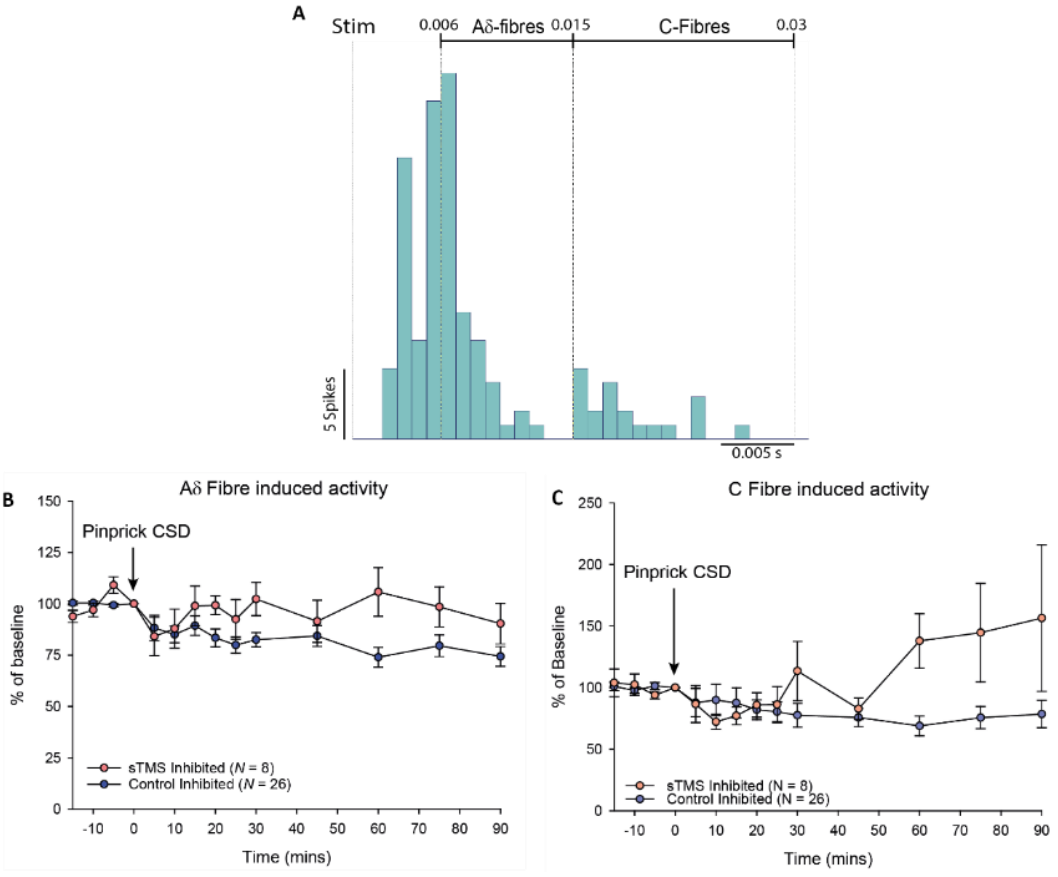

Figure 44: sTMS' affects thalamic trigeminally evoked neuronal activities response to CSD *(A) Representative example of trigeminally evoked thalamic activity, showing latency periods for Aδ fibre induced activity (0.006-0.015 sec post-stim) and C fibre induced activity (>15 sec post-stim) (B) There was a significant difference in Aδ fibre induced thalamic activity in the spontaneous activity inhibited groups of control vs sTMS pre-treatment (P = 0.014, Mann-Whitney) (C) There was a significant difference in C fibre induced thalamic activity in the spontaneous activity inhibited groups of control vs sTMS pre-treatment (P = 0.024, Mann-Whitney).*

CSD-induces a transient inhibition of spontaneous activity of the hypothalamus

To assess the cortico-hypothalamic connections spontaneous neuronal activity of the hypothalamus was recorded during a CSD. A total of 10 hypothalamic neurons in 10 animals were recorded. Only neurons that demonstrated stable spontaneous activity under baseline conditions were recorded during a CSD. The average spontaneous neuronal firing rates was 10.76 ± 1.82 spikes/sec.

Mechanically induced CSD caused an inhibition of cortical activity in hypothalamic neurons ($F_{2.85, 25.65} = 3.75$, $P = 0.025$), with maximum reduction of 36.5% occurring at 25 minutes post-CSD induction ($t_9 = 3.04$; $P = 0.014$, Fig. 45A). Cell firing recovered to baseline levels within 60 min post-CSD induction.

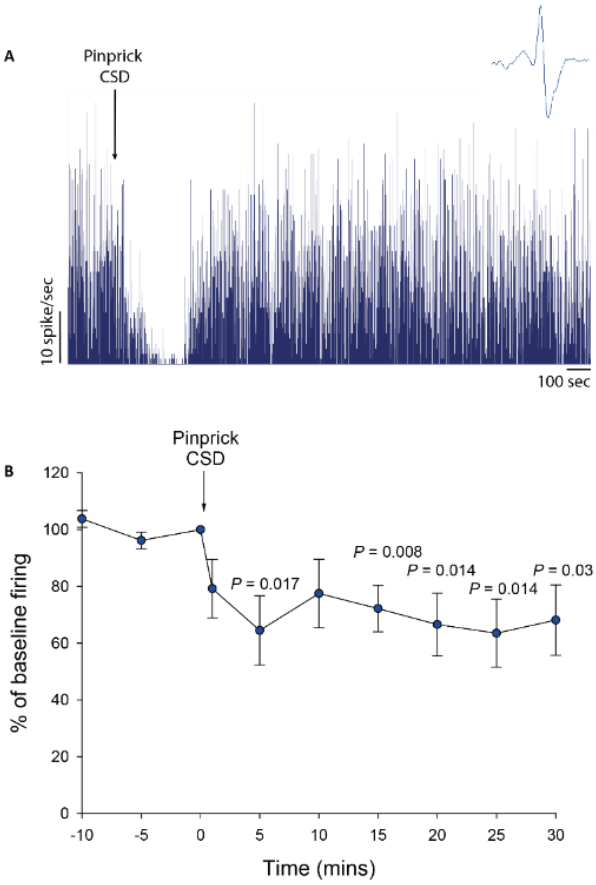

Figure 45: CSD inhibits spontaneous activity of hypothalamus *(A) Example of spontaneous hypothalamic activity during pinprick induced CSD showing representative bipolar action potential (B) Spontaneous hypothalamic activity following a pinprick induced CSD, inhibition of spontaneous activity was seen in the hypothalamus compared to baseline (P = 0.025; repeated measures ANOVA)*

Chapter 3:

Acute indirect effects of sTMS application on Thalamic and Hypothalamic Neuronal Activity

sTMS effect on spontaneous activity of the hypothalamus

To assess the cortico-hypothalamic modulation, spontaneous neuronal activity of the hypothalamus was recorded following cortical application of sTMS. Only neurons that demonstrated stable spontaneous activity for 15 minutes were recorded following CSD. A total of 24 hypothalamic neurons (in 6 animals) and 6 neurons (in 6 animals) were recorded in the sham (0 T) and sTMS (1.1 T) groups, respectively. Spontaneous neuronal firing rates were 2.39 ± 0.23 spikes/sec in the sham group and 1.52 ± 0.07 spikes/sec in the sTMS group.

Overall, 1.1T sTMS pulses applied to the occipital cortex caused inhibition of spontaneous hypothalamic activity (N = 6, 85.43% ± 3.28 of baseline) which was statistically significant (U = 8547, P = 0.012, r = -0.129, Fig. 46B). Each individual sTMS pulse also caused significant inhibition of hypothalamic activity.

The spontaneous neuronal firing rate was 1.95 ± 0.17 spikes/sec for the first sTMS pulse (U = 1986, P = 0.018, r = -0.197), 1.61 ± 0.12 spikes/sec for the second sTMS pulse (U = 1710.5, P = 0.001, r = -0.289), 1.36 ± 0.10 spikes/sec for the third sTMS pulse (U = 1586.5, P < 0.001, r = -0.323) and 1.33 ± 0.11spikes/sec for the fourth sTMS pulse (U = 1666, P < 0.001, r = -0.303, Fig. 46C).

Following electrophysiological recording brain tissue was collected, sectioned, and stained for dopamine cell marker tyrosine hydroxylase to locate the A11 hypothalamic nucleus to use as a histological landmark (Fig. 47B). The recording sites were found to be in the anterior, posterior, and ventromedial hypothalamic nuclei (Fig. 47A).

Chapter 3:

Acute indirect effects of sTMS application on Thalamic and Hypothalamic Neuronal Activity

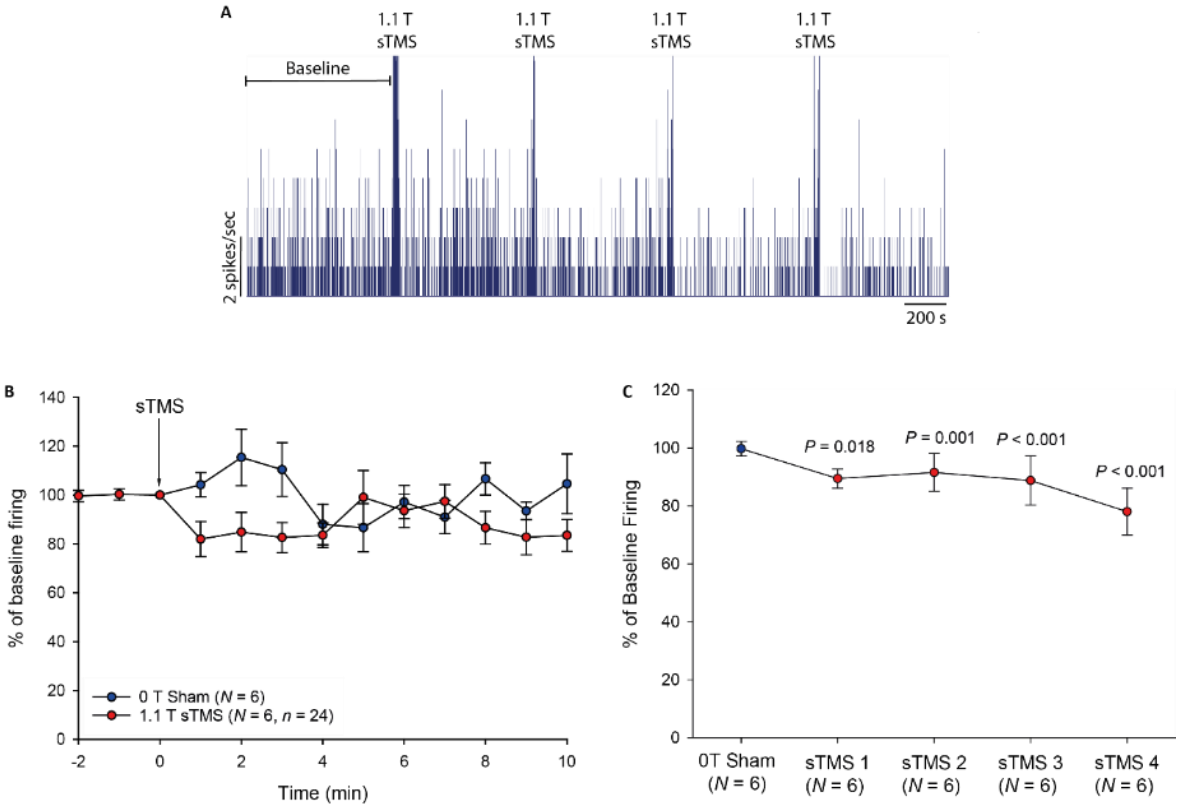

Figure 46: sTMS' effect on spontaneous activity of the hypothalamus *(A) Spontaneous activity following sTMS application, showing a transient inhibition compared to baseline (B) Spontaneous hypothalamic activity following 1.1 T sTMS pulse, inhibition of spontaneous activity was seen in the hypothalamus compared to baseline (P = 0.012; Mann-Whitney) (C) Averaged spontaneous hypothalamic activity for each individual subsequent sTMS pulse, inhibition of hypothalamic spontaneous activity was cumulative with multiple pulses (P = 0.018, P = 0.001, P < 0.001, P < 0.001; Mann-Whitney)*

Chapter 3:

Acute indirect effects of sTMS application on Thalamic and Hypothalamic Neuronal Activity

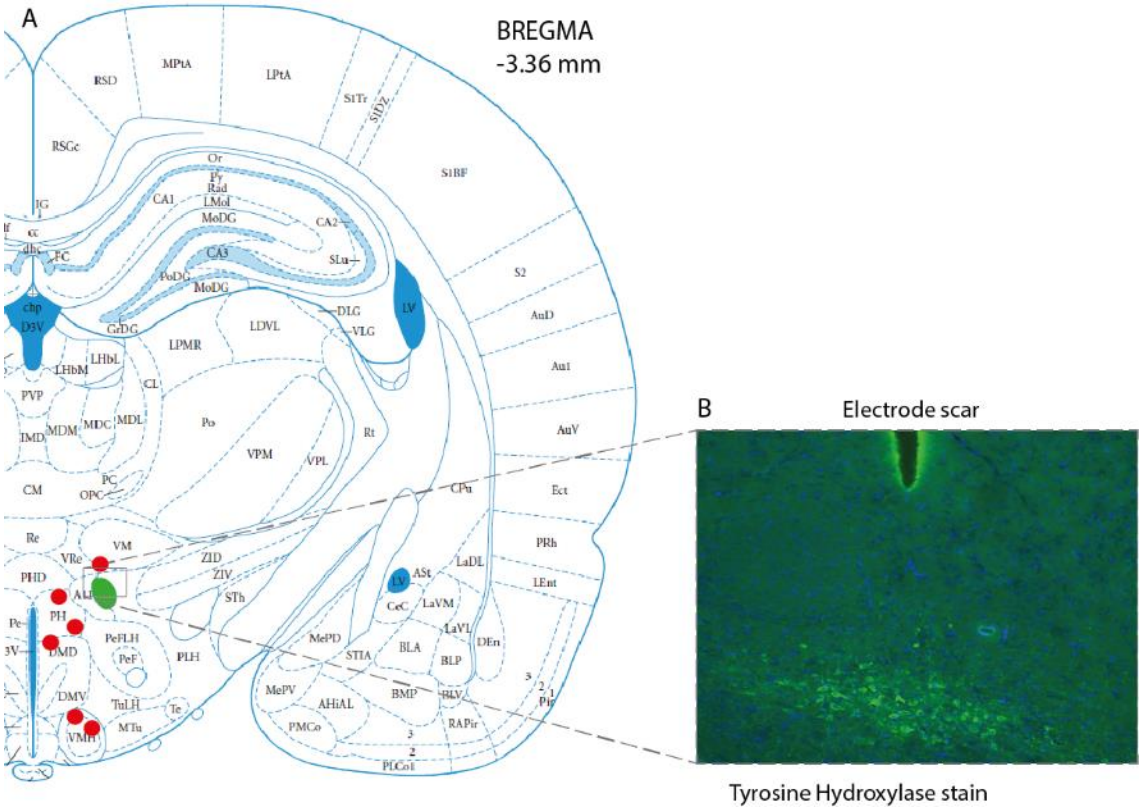

Figure 47: Hypothalamic recording sites *(A) Distribution of recording sites (red dots) with respect to the A11 nucleus marked in green used as histological landmark (B) Example image of brain slice following staining with tyrosine hydroxylase to show dopaminergic cells of the A11 nucleus and the electrode trace.*

Discussion

The primary purpose of this study was to identify the acute, indirect actions of single pulse transcranial magnetic stimulation (sTMS) application on the thalamic and hypothalamic neuronal activity. The data shown here demonstrates that activity of the thalamus and hypothalamus can be modulated by cortical events including CSD and sTMS.

Cortical modulation of the thalamic activity

Cortical application of 2x 1.1T sTMS was able to increase the activation threshold of 3rd order trigeminothalamic neurons of the VPM from electrical stimulation of 1st order trigeminal neurons innervating the superior sagittal sinus. This further elucidates how sTMS acts as an acute treatment for migraine attacks, increasing the peripheral stimulus required to activate the thalamus. This corroborates previous work by (Andreou et al., 2016) that similarly found that cortical sTMS was able to modulate VPM thalamic neuronal activity. They suggested this may be mediated by opioidergic systems as spontaneous and evoked third order neuron activity was blocked by narloxone. The likely site of action was suggested to be the cortex, blocking sTMS action at the site of application. This would also corroborate the interaction GABAergic interaction seen in the previous chapter, GABAergic cortical interneurons express mu (μ) opioid receptors (Taki et al., 2000). While the narloxone was administered systemically, mu (μ), delta (δ) and kappa (κ) opioid receptors are not found in the VPM thalamus (Mansour et al., 1987, George et al., 1994). Systemic narloxone could also be acting on ascending second order dorsal horn neurons (Mitchell et al., 2004) however you would expect that to produce an antinociceptive effect, contrary to what was seen. It would be interesting to investigate if local or cortical GABAergic blockade, negates the inhibition of the thalamus.

Descending cortico-thalamic afferents modulate thalamic activity via the thalamic reticular formation (Kim et al., 1997, Cox et al., 1997). Low-frequency cortical output activates GABAergic inhibition of thalamic activity (Crandall et al., 2015). TMS reduces cortical spontaneous activity, potentially through activation of cortical GABAergic

interneurons. TMS driven switch to low-frequency firing may drive the inhibition of the thalamus through recruitment of GABAergic modulation from the TRF. This would explain the increased thresholds for thalamic activity from stimulation of 1st order trigeminal neurons innervating the superior sagittal sinus. It would be interesting to investigate if there is increased activity in the TRF following sTMS application.

The thalamus exhibits two firing modes, tonic firing under normal conditions and burst firing following hyperpolarisation. Burst firing is controlled through activation of low voltage activated T-type calcium channels found on thalamocortical dendrites. An increase in cortically driven inhibition from sTMS application may also cause an increase in burst firing. This may be especially pronounced in migraine due to the hyperexcitability of the cortex promoting thalamic facilitation and preventing burst firing. It would be interesting to investigate if there is a change in the frequency of burst firing following application of cortical sTMS, especially in a cortically hyperactive model.

Cortical spreading depression was found to inhibit spontaneous activity of the thalamus, as well as response to Aδ and C-fibre afferent inputs in control and sTMS treated animals (Andreou et al., 2012). There was significantly greater extent of inhibition of spontaneous thalamic activity, in response to CSD, seen in sTMS pre-treated animals than control animals. This could suggest an additive effect, with thalamic spontaneous activity inhibited by both sTMS treatment and CSD. The proportion of thalamic cells pre-treated with sTMS that had inhibited spontaneous activity following CSD was increased when compared to the proportion inhibited in the sham control group (sTMS: 80% 8/10, Control: ~35%, 26/74). Similarly, there was a decreased proportion of thalamic cells with facilitated spontaneous activity following CSD when pre-treated with sTMS, compared to the proportion facilitated in the sham control group (sTMS: 20% 2/10, Control: ~35%, 26/74). This could be a potentially interesting finding, supporting the inhibition seen with the increased activation thresholds. However, there was a large difference in the group sizes (sTMS: $N = 10$, control: $N = 74$), therefore further experiments would be required to address this and determine if the altered inhibited/facilitated proportions were the result of the sTMS treatment or simply a sampling artefact.

There was a significant difference in trigeminally evoked thalamic activity following CSD, between control and sTMS pre-treated animals. In control animals the change in Aδ and C fibre evoked activity during CSD mirrored the change in spontaneous activity, therefore decreased spontaneous activity, also decreased Aδ and C fibre evoked activity. However, in sTMS pre-treated animals, although the extent of inhibition of spontaneous activity was greater than control following CSD, Aδ and C fibre evoked thalamic activity was not significantly altered following CSD. This could suggest that sTMS reduces the effect of CSD on thalamic activity.

Cortical modulation of the hypothalamic activity

The results shown here suggest that spontaneous activity of the hypothalamus can be inhibited by cortical events, such as CSD and sTMS applied to the visual cortex. Inhibition of hypothalamic activity by sTMS appeared to be cumulative with multiple subsequent sTMS pulses producing increased inhibition of the hypothalamus. Given the hypothalamus' role as a potential trigger for migraine attacks, corticohypothalamic cumulative modulation may suggest a potential mechanism by which sTMS reduces headache days as a preventative treatment, reducing hypothalamic excitability and thus preventing migraine attack initiation.

Multiple hypothalamic nuclei were recorded from including the A11, anterior, posterior, and ventromedial hypothalamic nuclei. The variation in recording site location is primarily due to the small size of the hypothalamus deep within the brain. Additionally, the hypothalamic nuclei have much lower neuronal spontaneous firing rates compared to other brain structures (such as the cortex) making spontaneous activity recording more challenging. Finally, the lack of a para-orbital receptive field (as in the thalamic recordings) removed the ability to confirm and correct electrode placement while the experiment was ongoing. The later confirmation meant more experiments had to be performed and discounted after the fact, squandering available time and resources. Further experiments would be required to record from each individual hypothalamic nuclei to determine if cortical sTMS application has a modulatory effect.

Potential individual nuclei of interest to investigate further include; the A11 dopaminergic nucleus, and the tuberomammillary nucleus (TMN). The A11 nucleus is a region of the hypothalamus containing dopaminergic neurons, and the sole source of dopaminergic modulation of the spinal cord (Holstege et al., 1996). The tuberomammillary nucleus (TMN) is a posterior hypothalamic nuclei and the sole source of histamine within the brain is involved with sleep-wake states (Haas and Panula, 2003, Takahashi et al., 2006). This has led to the suggestion that it may be involved with the peak in migraine attacks mid-morning morning (Fox and Davis, 1998a, Alstadhaug et al., 2008).

Conclusion

In conclusion, in addition to directly modulating neuronal activity in the cortex, sTMS is also modulates neuronal activity in nuclei related to migraine, particularly activity of the thalamus. This modulation of these cortical pathways further elucidates how sTMS treatment achieves both an acute and preventative treatment effect for migraine.

Chapter 4: Long term application of sTMS

Chapter 4:

Long term application of sTMS

Introduction

Singe pulse transcranial magnetic stimulation (sTMS) was initially introduced as an acute treatment following a randomised, sham control clinical trial (Lipton et al., 2010) and NICE approval. Anecdotal reports from patients using sTMS began to indicate an overall reduction of headache frequency, suggesting a potential role of sTMS in the prevention of migraine. This led to post-market studies that looked at the efficacy of sTMS as a preventive treatment which found it effective (Bhola et al., 2015, Starling et al., 2018). The mechanism of action of sTMS as a preventive treatment has not been previously studied.

One of the major issues with the currently available acute medications for migraine is medication overuse headache (MOH). A secondary headache disorder (8.2 in ICHD-3) MOH is defined as:

> *"Headache occurring on 15 or more days/month in a patient with a pre-existing primary headache and developing as a consequence of regular overuse of acute or symptomatic headache medication (on 10 or more, or 15 or more days/month, depending on the medication) for more than three months."* (IHS, 2018).

Paradoxically, by overusing acute medications to control a pre-existing headache, a feedback loop is created where a headache leads to medication treatment, leading to further headaches (separate from the initial condition), leading to further intake of analgesics, and so on. A headache caused by analgesics was first observed in Swiss pharmaceutical factory workers given samples of phenacetin in the 1950's (Moeschlin, 1957). The median worldwide prevalence of MOH is around 1-2% (Zwart et al., 2003), however this varies by country with some studies finding up to approximately 7%. Although these differences in prevalence are likely due to differences in the definition of the disorder and local prescribing practices. It is worth mentioning though that a higher percentage of chronic migraine patients referred to tertiary centres may suffer from MOH.

The current recommended treatment is detox from analgesic use for several weeks until the MOH resolves (Evers and Jensen, 2011). Additionally, there is mixed evidence for the efficacy of prophylactic treatments in MOH (Sandrini et al., 2011, Grande et al., 2011). The problem with MOH in a clinical setting is that the pre-existing headache

remains, thus temporarily withdrawing analgesics will result in the main condition being untreated. Additionally, withdrawal can itself temporarily produce further headaches, making the patients ill-disposed to detoxify. Detoxification can be aided with the use prophylactic treatments that are equally effective in MOH and non-MOH patients, such as BoNT/A (Andreou et al., 2018).

All acute medications taken for migraine, including; triptans, NSAIDs (Starling et al., 2011), paracetamol and ergots, appear to be able to produce MOH, although the precise features differ depending on the overused medication. Triptan-induced MOH is suggested to be induced after approximately 1.7 years of 18 doses per month and produced worsening of frequency and intensity of existing migraine headaches (Limmroth et al., 2002). NSAID-induced MOH was produced after 4.8 years and 114 doses per month, this produced headache alike to daily tension-type headache. Finally ergot-induced MOH was induced after 2.7 years of 37 doses per month and produced a TTH-like headache (Limmroth et al., 2002). In the EU triptans are the most overused drug (61%) followed by NSAIDs.

The overuse of analgesics is not limited to just migraine patients, many chronic pain patients take daily analgesic medication to facilitate normal lifestyles. However, even when analgesics are being taken for other conditions, patients with a history of migraine are more likely to develop MOH (Bahra et al., 2003).

Beyond an interaction between excessive use of analgesics and a susceptible patient, it is unclear exactly what the pathophysiology of MOH is. Imaging of voxel-based morphometry studies in MOH patients found alterations in grey matter volumes in areas associated with pain processing. This includes increased grey matter volume in the left temporal lobe, thalamus ventral striatum, and periaqueductal grey and decreased grey matter volume in the orbitofrontal cortex and left middle occipital gyrus, anterior cingulate cortex and insula (Riederer et al., 2012, Lai et al., 2016, Chen et al., 2017). The increase in grey matter of the periaqueductal grey especially seams to play a significant role in MOH as it positively correlates with the disability score of the patient (Riederer et al., 2012) and shows a decrease in line with the degree of clinical improvement following detoxification (Riederer et al., 2013).

It has been proposed that there is overlap in the pathophysiologies of MOH and addiction. Brain imaging studies of MOH patients have shown reduced activity in the substantia nigra and ventral tegmental areas and increased activity in the ventromedial prefrontal cortex (Ferraro et al., 2012). These dopaminergic midbrain structures make up the mesocorticolimbic system, known to play a role in the motivation and rewarding of behaviours and implicated in addiction (Goldstein and Volkow, 2002). Allele 10 of dopamine transporter gene (*SLC6A3*) was significantly lower in MOH patients[4] than episodic migraine without aura patients, however this was not found to be significantly different than healthy controls (Cevoli et al., 2006). Single nucleotide polymorphism of the brain derived neurotropic factor (BDNF) gene (*Val66Met*), which reduces BDNF activity, has previously been linked to substance abuse (Lang et al., 2007, Gratacòs et al., 2007) was found to be a predictor of analgesic drug consumption in medication overuse headache, particularly combination drug use (Di Lorenzo et al., 2009).

Animals exposed to high doses of sumatriptan (0.6 mg/kg/day) and naratriptan (200 µg/kg/day) developed cutaneous allodynia and sensitisation of the primary afferent trigeminal fibres, with increased response to Nitric oxide (NO) donor and increased plasma levels of CGRP. Termination of triptan exposure resolved sensory sensitivities, however trigeminal fibres still had increased expression of CGRP (De Felice et al., 2010). This expression of CGRP may be driven by abnormal activation of trigeminal $NaV_{1.9}$ by NO (Bonnet et al., 2019). Animal models exposed to chronic triptans have also shown downregulation of $5\text{-}HT_{1B/D}$ receptors in the trigeminal ganglion and basilar artery (Reuter et al., 2004).

Headache is one of the most common adverse effects reported in clinical studies with sTMS as a treatment for migraine, in ~2% of patients. However, none of the adverse events reported, including headache, were considered to be clinically different to the sham treatment group (Lipton et al., 2010, Starling et al., 2018). A recent study found that single pulse TMS applied to Broca's area and the primary motor cortex induced scalp pain. This was believed to be due to stimulation of the peripheral nociceptors rather than top-down modulation. The stimulus required to produce scalp pain was significantly lower than the

[4] Called "Chronic daily headache with associated drug abuse"

motor threshold (Tani et al., 2020). It is currently unclear if any of the acute neuromodulation treatments for migraine, including sTMS, induce a form of "machine overuse headache", similar to MOH.

Prophylactic treatments for migraine aim to prevent the occurrence of migraine attacks. Several prophylactic options are recommended by National Institute for Health and Care Excellence (NICE) as first-line treatments including; beta-blockers (propranolol), tricyclic antidepressants (amitriptyline) and anti-epileptics (topiramate) (Kennis et al., 2013). BoNT/A injections are recommended as a tertiary prophylactic treatment in patients that have tried and failed at least 3 previous prophylactic options. Recently there have been exciting developments in migraine prophylaxis with the calcitonin gene related peptide (CGRP) monoclonal antibodies, however none are currently approved by NICE for prescription in the NHS. As of 2014 single-pulse transcranial magnetic stimulation (sTMS) has been approved for the treatment and prevention of migraine by NICE in the UK and Food and Drug Administration (FDA) in the USA.

Although limited data on the effects of long-term application of sTMS, in migraine patients suggest its usefulness in the reductions in monthly headache days (Bhola et al., 2015, Starling et al., 2018), to date, no pre-clinical studies have been conducted into the functional effects of long-term application of sTMS in animal models. In order to assess the effect of long-term application of sTMS on sensitisation of peripherally driven pathways, behavioural testing of mechanical pain thresholds of the periorbital region (innervated by the ophthalmic branch of the trigeminal nerve) was used in animals receiving daily sTMS treatment. This involves applying a range of flexible filaments (von Frey filaments), that each deliver a specific weight, to the V1 dermatome and monitoring for stereotypical behavioural responses (such as flinching away from the filament). Thereby, determining a threshold weight that provokes a pain behaviour. This protocol has previously been widely used by studies modelling MOH in animals exposed to long-term, high levels of acute migraine medications, such as sumatriptan (De Felice et al., 2010, Green et al., 2014). The behavioural testing was supplemented by examining activation of second order Trigeminocervical complex (TCC) neurons following trigeminovascular stimulation, by means of Fos immunoreactivity in the TCC. By comparing animal groups given active or sham sTMS over the course of 1 month, the

primary aim was to determine if long-term application of sTMS causes any peripheral sensitisation of second order trigeminal neurons. Any developed sensitisation may indicate the possibility of developing MOH-like complications when using sTMS over long periods.

Following the month of daily sTMS application, electrophysiological testing was carried out to explore any cumulative long-term effects of sTMS. Focusing on the brain structures previously show to be affected by acute application of sTMS, the cortex and thalamus, and following the protocols set out in previous chapters.

The focus of this chapter is two-fold, firstly to investigate if daily sTMS application causes sensitisation of second order neurons, which may suggest a neuromodulation mechanism equivalent to MOH (machine overuse headache). Secondly, to examine if the acute effects seen in previous chapters are cumulative, thus shedding light on how sTMS acts as a prophylactic medication.

Chapter 4:
Long term application of sTMS

Methods

Animals and ethical approvals

All experiments were performed in accordance with the United Kingdom Home Office Animals (Scientific Procedures) Act (1986). Experiments were carried out in adult male Sprague Dawley rats (*N* = 37, 150-400 g; Charles River, UK). Rats were housed on a 12/12-hour light/dark cycle with food and water available *ad libitum*. Behavioural experiments were performed on awake unrestrained animals. Electrophysiological experiments were performed under general anaesthesia and terminated by anaesthesia overdose and/or cervical dislocation.

Daily sTMS Application and Peri-orbital Mechanical Pain testing

For up to 7 days prior to the start of testing, animals were gradually habituated to; the experimenter, the testing room, the testing protocol, and the treatment protocol. Throughout the treatment period weight of the animals was monitored for animal welfare and external signs of stress (i.e., animal weight and porphyrin secretion) were monitored.

Awake unrestrained animals were placed in 22 x 16.5 x 14 cm transparent Perspex von-frey box and allowed to habituate for 20-30 minutes prior to testing. The mechanical pain threshold was determined by testing the periorbital region using TouchTest von frey filaments (North Coast, USA). Starting from the lightest 2.36 (0.02 g) and increasing to a maximum of 5.07 (10 g) (Table 7) the filaments were applied to the periorbital region for approximately 1-2 seconds (Fig. 48A). Between each stimulus, a rest period of a minimum of 5 minutes was allowed to prevent sensitisation. The periorbital region was swapped between each stimulus, however in some cases, due to the position of the animal in the box, this was not possible. Stimulation was increased until a stereotypical flinching of the head away from the filament was elicited, at which time the stimulus was repeated to confirm response, with final confirmation achieved by dropping to the previous sub threshold stimulus to confirm it does not cause the response. Behavioural testing was performed in the morning of; days 1, 3, 5, 7, 9, 11, 15, 20, 25, and 30 (Fig. 48B).

Chapter 4:

Long term application of sTMS

Animals were split into two treatment groups. Group 1 received 4x 1.1 T (600 V) sTMS pulses daily to the posterior of the head over the occipital cortex (*N* = 25). Group 2 was a sham control group which received daily 4x 0 T (0 V) sTMS treatments (*N* = 20). In the first series of experiments animal were housed 5 per cage, in mixed treatment cages, however, in subsequent experiments animals were housed 4 per cage, all of which received the same treatment. During behavioural testing the experimenter was blinded to which treatment each animal received. Unblinding was performed upon completion of experiments.

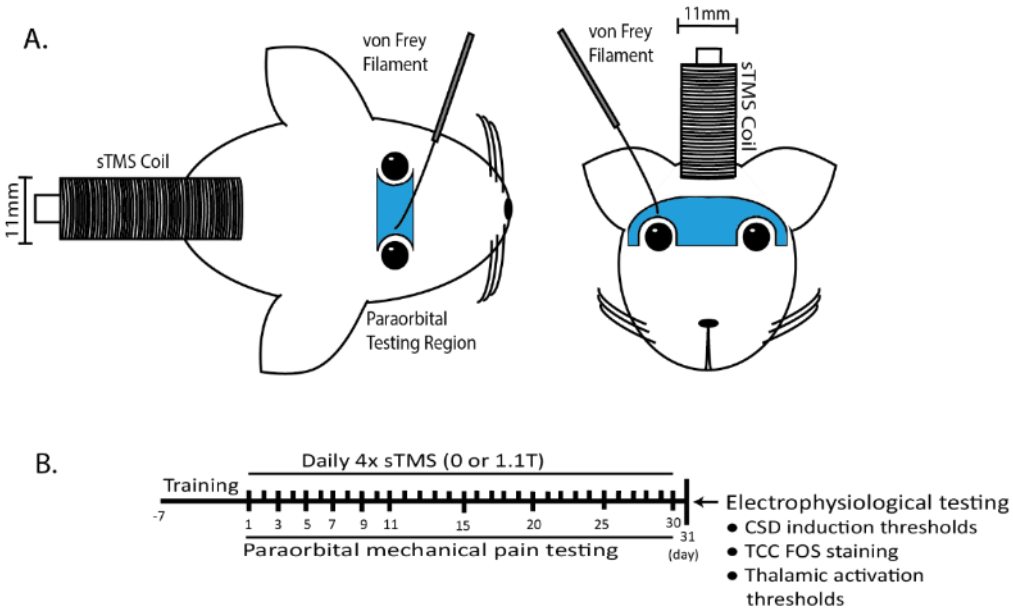

Figure 48: Long term sTMS application and periorbital mechanical pain threshold testing *(A) Experimental setup in the rat model: sagittal and frontal views, showing TMS application site and periorbital testing site (B) Diagram illustrating the experimental protocol employed in the long-term sTMS application model, 0 T sham or 1.1T sTMS was applied daily for 30 days, while periorbital mechanical pain testing was performed on days 0, 1, 3, 5, 7, 9, 11, 16, 20, 25 and 30.*

Table 7: Touch-Test™ Sensory Evaluator Chart, showing range of weights used

Product Number	Evaluator Size	Target Force*	Representation	Hand & Dorsal Foot Thresholds	Plantar Thresholds
NC12775-02	2.36	0.02	Green	Normal	Normal
NC12775-03	2.44	0.04			
NC12775-04	2.83	0.07			
NC12775-05	3.22	0.16	Blue	Diminished Light Touch	
NC12775-06	3.61	0.4	Blue Purple	Diminished Light Touch Diminished Protective Sensation	Diminished Light Touch
NC12775-07	3.84	0.6			
NC12775-08	4.08	1	Purple Red	Diminished Protective Sensation	Diminished Light Touch
NC12775-09	4.17	1.4			
NC12775-10	4.31	2			
NC12775-11	4.56	4		Loss of Protective Sensation	Diminished Protective Sensation
NC12775-12	4.74	6	Red	Loss of Protective Sensation	Diminished Protective Sensation Loss of Protective Sensation
NC12775-13	4.93	8			
NC12775-14	5.07	10			

**Individually calibrated within a 5% standard deviation.*

Surgery

At the end of the 30-day trial, animals were assigned at different experimental procedures:

a. Trigeminovascular stimulation and Fos staining in TCC
b. CSD induction threshold
c. Third order thalamic neurons activation threshold

To separate the acute effects from the cumulative long-term effects, a minimum of 24 hours break was allowed between the final sTMS application and the electrophysiological experiments.

For these experiments, all animals were anesthetised with intraperitoneal injection of 60 $mg.kg^{-1}$ pentobarbital sodium (Merial, UK). and were physiologically monitored as previously described in chapter 2 (Methods). The animals were then fixed on a non-magnetic stereotaxic frame (Kopf instruments). Craniotomies were performed and dura mater incised to expose the occipital and parietal cortex for electrophysiological recordings/stimulations. For the trigeminovascular stimulation experiments the superior sagittal sinus (SSS) was exposed and electrically stimulated.

A. Superior Sagittal Sinus (SSS) Stimulation and FOS staining

To test activation of second order neurons, the superior sagittal sinus (SSS) was exposed and stimulated with platinum wire stimulating electrodes with horizontal tips (2 mm approx.), placed atop (touching but not compressing) the sinus using a micromanipulator (Fig. 49A). Trigeminal primary afferent fibres were activated by stimulating them with square wave electrical pulses (0.5 Hz, 0.1 ms duration, 30 V) for a period of 2 hours. Following stimulation, animals were perfused intracardially with paraformaldehyde (PFA). TCC tissue was removed and post-fix in PFA following preservation in a 30% sucrose solution, before preserved tissues were frozen in OCT compound (361303E, VWR Chemicals, Belgium) and sectioned into 30 µm slices using a UV cryostat (CM1860, Leica, Germany) and placed onto Superfrost microslides slides (3610108, VWR Chemicals, Belgium).

TCC slides were stained using a rabbit anti-cFOS monoclonal primary antibody (SC52, Santa Crux Biotechnologies, USA) 1:500 dilution for 24 hours. This was followed by incubation with a goat anti-rabbit secondary antibody (BA1000, Vector Laboratories, Canada) at 1:250 dilution for 90 minutes. Finally DAB Substrate Kit (SK-4100, Vector Laboratories, Canada) was applied to the slides for 90 minutes. FOS staining on TCC slides was counted in a blinded fashion using an AxioSkop microscope (Zeiss, Germany). The methods for counting Fos protein-stained nuclei have been described (Park et al., 2014, Benjamin et al., 2004). Briefly, morphological appearance of the sections under 5x brightfield microscopy was used to distinguish and group FOS counts between the trigeminal nucleus caudalis (TNC), C1 and C2 regions of the TCC. Dark brown to black

cells were considered to be FOS-positive. At each level, four randomly selected sections were assessed and an average of all cells per section for each level was calculated in order to investigate the distribution pattern at each nucleus, without prior knowledge of treatment for each animal. Representative brightfield images were taken using an Axio Imager.Z1 microscope with a 10x lens (Zeiss, Germany).

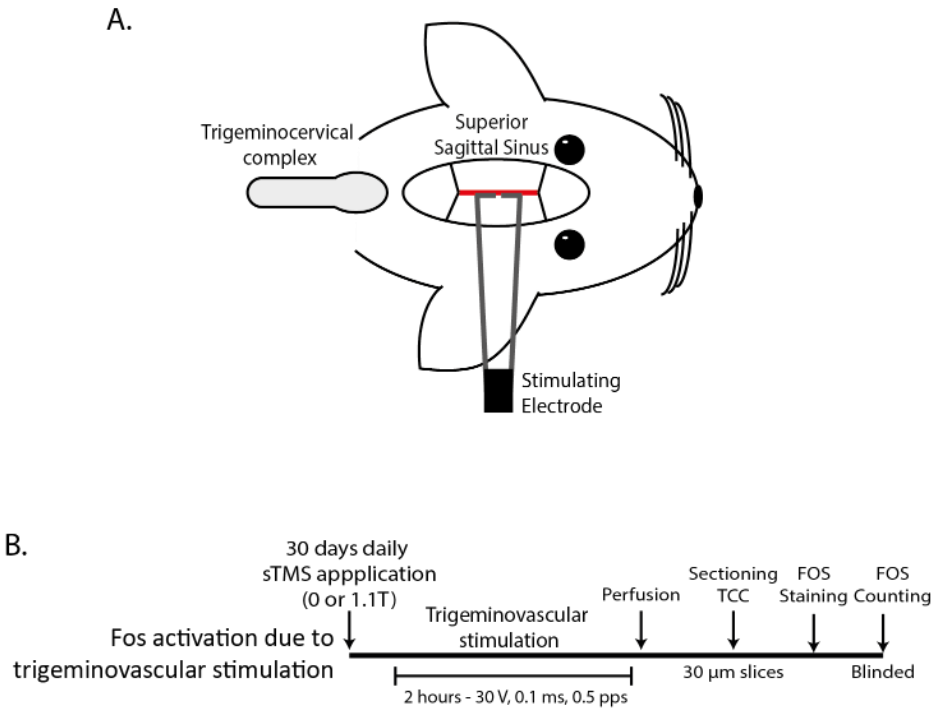

Figure 49: Long term application of sTMS on activation of second order neurons *(A) Experimental setup in the rat model: superior sagittal sinus (SSS) stimulated for 2 hours at 30 V, 0.1 ms and 0.5 pps. Animals were then perfused and the TCC was collected, sectioned and stained for cellular activation marker FOS (B) Diagram illustrating the experimental protocol employed in electrically stimulating trigeminal primary afferents innervating the SSS in animals given daily application of sTMS at 0T (sham stimulation) or at ~1.1T for 30 days prior to testing.*

B. Electrically induced CSD Threshold

To identify if 30 days of sTMS application increases the threshold of activation for CSD induction, the migraine aura model of electrically induced CSD was utilised. The CSD induction threshold was found in animals pre-treated with 30 days of 4x sTMS pulses (~ 0 T or 1.1 T; $N = 8$ per group) over the visual cortex. A small cranial window was drilled over the frontal cortex and another prepared over the visual cortex. The latter was used to electrically induce CSD as described below. Cortical steady potential was recorded via

1.5 mm borosilicate glass microelectrode with a tip diameter of 1 - 2 µm containing 3 M NaCl, inserted to a depth of 50 - 100 µm in the frontal cortex. The microelectrode was coupled to a reference Ag/AgCl electrode placed in contact with exposed neck muscle. The signal was amplified (NL102G head-stage, NL102 DC amplifier) and displayed on a computer. As a secondary measure, blood flow changes caused by the CSD wave, were monitored via laser doppler (Moor Instruments) using a 1.5 mm (OD) optical probe (VP3, Moor Instruments, USA). The probe was attached to a laser doppler perfusion and temperature monitor (DRT4, Moor Instruments, USA) and displayed on a computer (Fig. 50A). Warm mineral oil was used to prevent exposed areas drying.

CSD was induced electrically, using a constant current isolated stimulator (DS3, Digitimer) with a concentric bipolar stimulating electrode (tip diameter 25 µm, FHC, USA) inserted to a depth of ~100 µm into the visual cortex. (Fig. 50A). CSD threshold was found by increasing duration (50, 100, 200, 400 µs) and amplitude (0.5, 1, 2, 3, 4, 5 mA) of electrical stimulus until a CSD wave was triggered. (Fig. 50B).

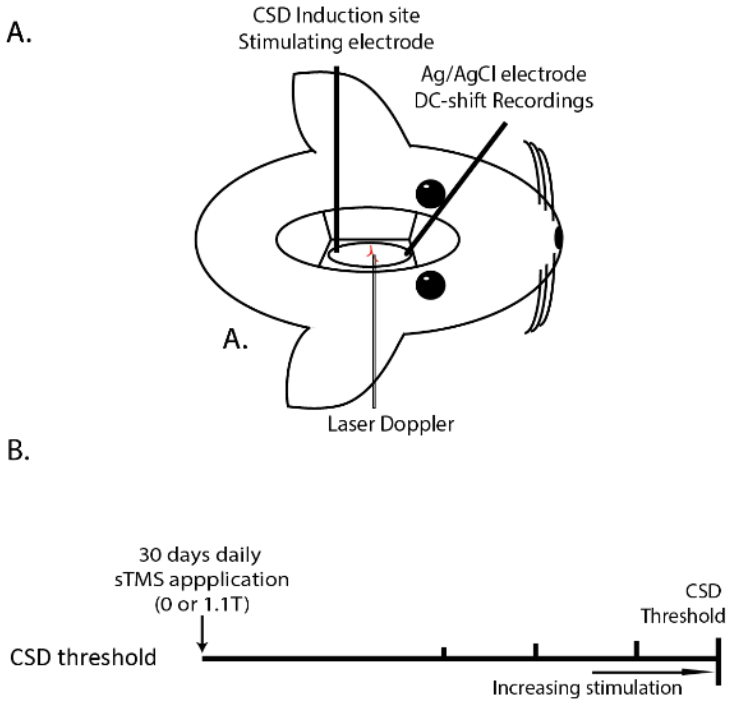

Figure 50: Long term sTMS application on CSD induction threshold *(A) Experimental setup in the rat model: Cortical steady potential (DC-shift) was recorded in the visual cortex using a Ag/AgCl glass microelectrode. CSD was electrically induced at the visual cortex. The sTMS coil was positioned above the cortex, close to the CSD induction site (B) Diagram illustrating the experimental protocol employed in the electrically induced CSD model of migraine, in animals given daily application of sTMS at 0T (sham stimulation) or at ~1.1T for 30 days prior to testing*

C. Electrical activation threshold of third order neurons in the ventroposteromedial thalamic nucleus (VPM)

A cranial window was drilled over the approximate location of the ventroposteromedial thalamic nucleus (VPM), also exposing the superior sagittal sinus. A glass-tungsten recording electrode was stereotaxically lowered into the VPM thalamic nucleus as previously described (Fig. 38).

The trigeminal primary afferent fibres innervating the SSS were stimulated by lowering a pair of platinum wire stimulating electrodes onto the SSS using an electrode manipulator (1460, Kopf Instruments, USA). Care was made to only make contact with the sinus and not the cortex (Fig 51A). Upon identification of a third order neurons, activated in response to a light stroke of the contralateral ophthalmic receptive field, neuronal activity in the VPM thalamic nucleus was simultaneously recorded. The voltage of the stimulus over the SSS was increased between 5 - 40 V until a consistent action potential of thalamic third order neurons was seen. Thresholds were confirmed by reducing the stimulus to the previous sub-threshold stimulation to confirm the absence of an action potential, before returning to threshold stimulation (Fig. 51B). Thalamic stimulation thresholds were found in 15 separate cells per animal (N = 5) in both 0 T sham and 1.1 T sTMS group.

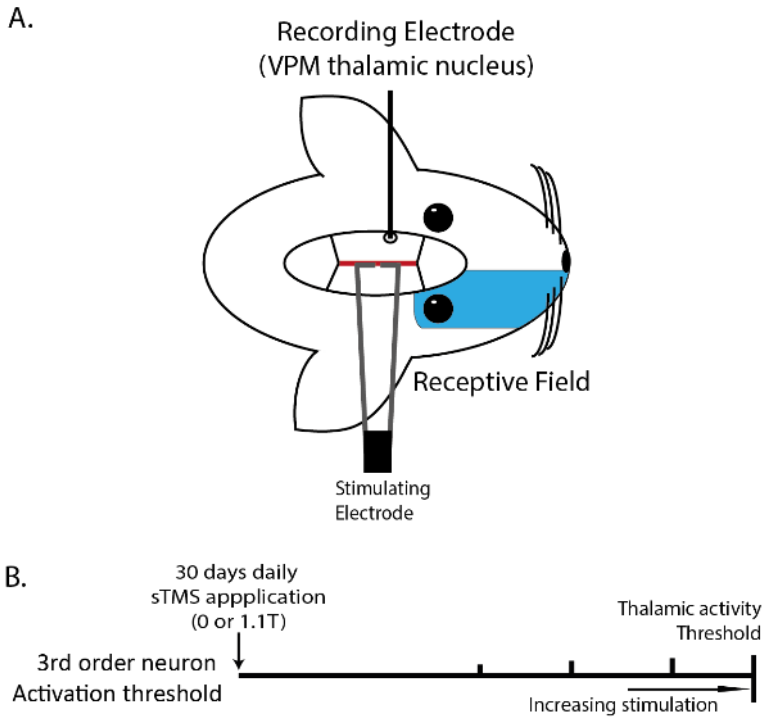

Figure 51: Long term application of sTMS on the activation threshold of 3rd order thalamic neurons *(A) Experimental setup in the rat model: primary trigeminal fibres innervating the superior sagittal sinus were stimulated with a pair of platinum electrodes, in order to induce action potentials in third order neurons in the VPM thalamic nucleus, which were recorded with a glass tungsten electrode (B) Diagram illustrating the experimental protocol employed in these experiments, in animals given daily application of sTMS at 0T (sham stimulation) or at ~1.1T for 30 days prior to testing*

CSD induction and recordings from thirds order VPM neurons

To investigate if long-term sTMS had any effect on the CSD-induced changes on third order VPM neurons, spontaneous activity of the VPM thalamic nucleus was also recorded in response to pinprick induced cortical spreading depression (Fig 52A). Baseline spontaneous neuronal activity was recorded for 15 minutes prior to a needle prick initiation of CSD. Neuronal activity was recorded for 30 minutes post-CSD, initial activity was averaged in 1-minute bins for 10 minutes post-CSD, followed by 5-minute bins up to 30 minutes (Fig. 52B). All data were expressed as a mean percentage of baseline ± SEM (SPSS version 23.0; SPSS Inc., USA).

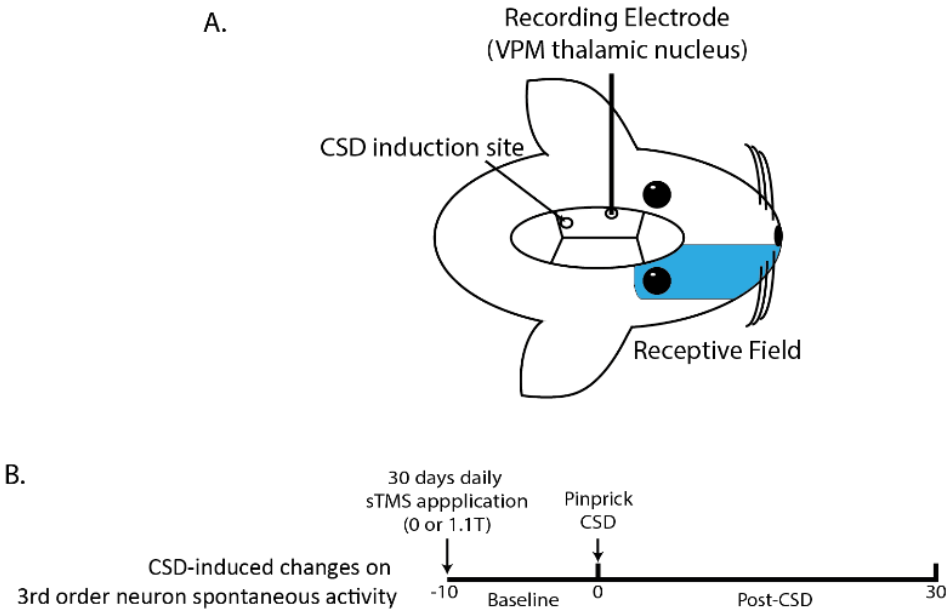

Figure 52: Long-term application of sTMS on 3rd order neurons response to cortical CSD *(A) Experimental setup in the rat model: glass-tungsten recording electrode placed stereotaxically in the VPM thalamic nucleus, confirmed with peri-orbital Dural receptive field, CSD initiated via pinprick to occipital cortex (B) Diagram illustrating the experimental protocol employed in the spontaneous thalamic activity response to CSD, in animals given daily application of sTMS at 0T (sham stimulation) or at ~1.1T for 30 days prior to testing*

Statistical Analysis of Peri-orbital Mechanical Pain testing

Statistical analysis of the data was completed using SPSS Statistics 23 (IBM, USA). ANOVA for repeated measures was used followed by Student's paired *t*-test. Data are expressed as mean percentage of the baseline activity ± SEM and significance was assessed at the $P < 0.05$ levels.

Statistical Analysis of Electrophysiological testing

Effects of long-term application of sTMS between sham and 1.1 T sTMS groups, for all electrophysiological tests were analysed using the Mann Whitney test. Significance was assessed at the $P < 0.05$ level. All data are expressed as the median value (FOS count) and interquartile range (IQ; 25% – 75% range) (SPSS version 23.0; SPSS Inc., USA).

Graphs

Graphs of all analysed data were produced in SigmaPlot 14.0 (Systat software Inc, USA).

Results

Long-term sTMS treatment and periorbital mechanical pain thresholds

The periorbital mechanical pain thresholds were found in animal groups receiving daily 0T sham-treatment (N = 8, 5.06 ± 0.65 g) and 4x daily 1.1 T sTMS treatment (N = 8, 6.10 ± 0.61 g). Both 0 T sham ($F_{2.26, 15.82}$ = 7.636, p = 0.04) and 1.1 T sTMS ($F_{3.51, 24.57}$ = 13.011, P < 0.001) groups showed a significant reduction in pain thresholds from a baseline of 9.38 g ± 0.44 to 3.26 g ± 0.63 at day 11. However, there was so significant difference between the sham and sTMS treatment groups (F_{78} = 1.337, P = 0.251, Fig. 53B).

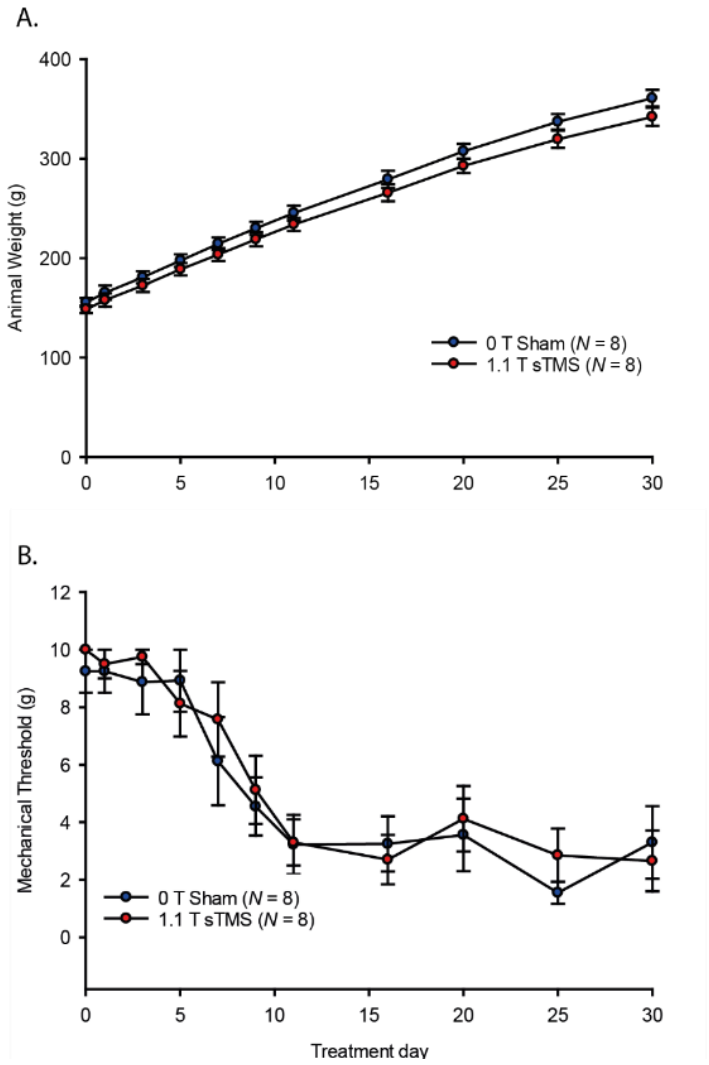

Figure 53: Daily 1.1 T sTMS application has no difference to para-orbital mechanical pain thresholds *(A) No difference in weight gain between sham and sTMS groups was observed during the 30 days sTMS stimulation period (B) Daily treatment of sTMS for 30 days (both 0 T sham and 1.1 T sTMS) caused a reduction in mechanical pain thresholds from baseline (sham - p = 0.04, sTMS - p < 0.001; rmANOVA). However, there was no significant difference between treatment groups (p = 0.251; students T-test).*

Actions of long-term sTMS treatment on Fos activation in response to trigeminovascular activation

At the end of the 30 days sTMS treatment period, animals in both treatment groups (0 T and 1.1 T) received trigeminovascular stimulation by electrically stimulating trigeminal afferents innervating SSS. Subsequent activation of the second order neurons in the TCC was investigated with staining for the cellular activation marker FOS (Fig 54A). There was no significant difference between the 0 T sham treated group (N = 4, median: 48.5, IQ: 6.75 – 81.25) and the 1.1 T sTMS treated group (N = 7, median: 44.5, IQ: 19.0 – 67.0) (U = 52.0, P = 0.785, r = -0.058) (Fig 54B).

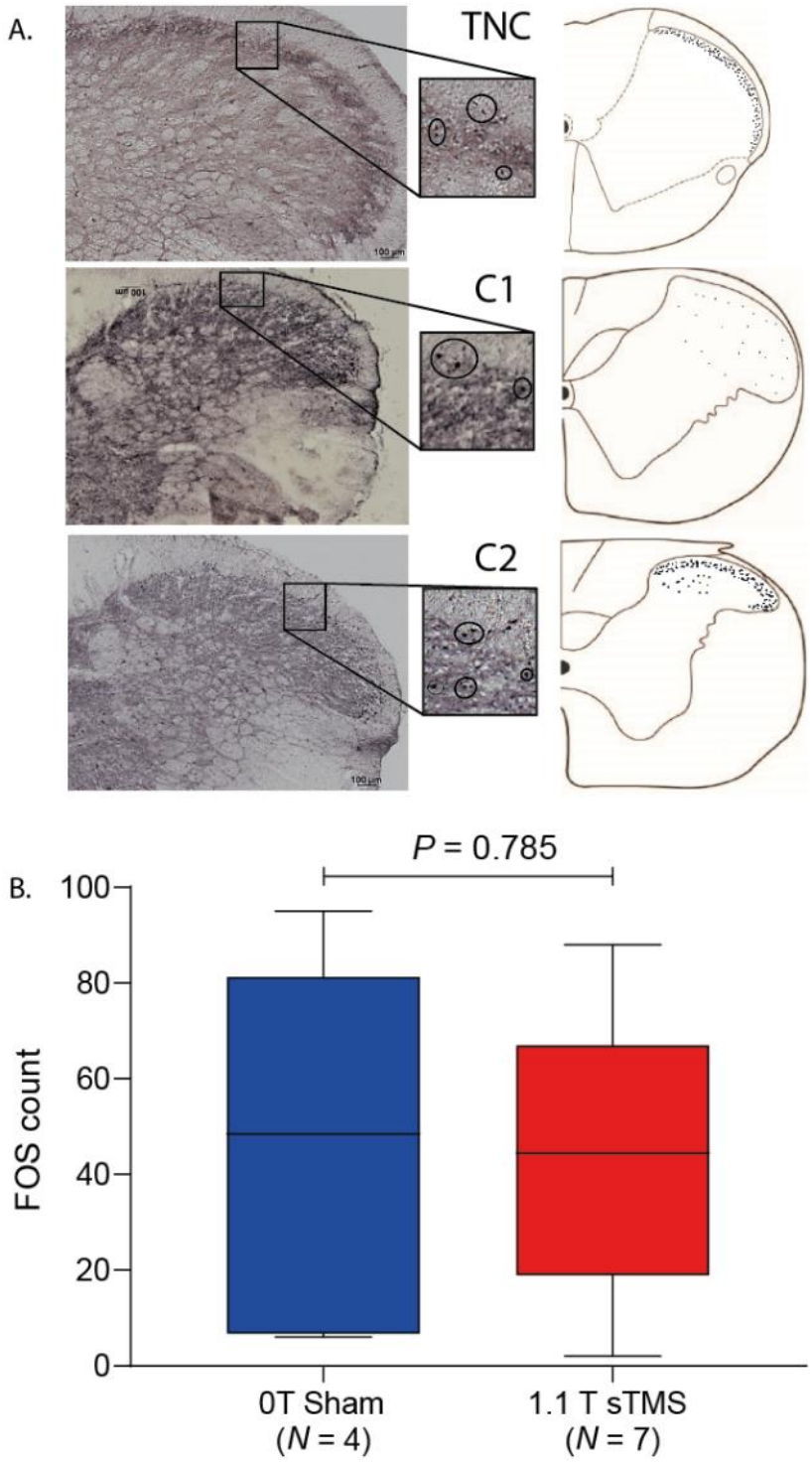

Figure 54: Daily 1.1 T sTMS application had no significant difference on the activation of second order neurons in the trigeminocervical complex *(A) Examples of trigeminocervical complex slices stained with FOS to show activation of second order neurons, and diagrams illustrating location of Fos-positive cells (B) Daily sTMS for 30 days at ~1.1 T had no significant effect on the number of FOS-positive cells in the TCC (P = 0.785, Mann-Whitney test) vs 30 days of 0 T sham application.*

Actions of long-term sTMS treatment on electrically induced CSD threshold

The electrical stimulation threshold for CSD was determined in the sham-treated group (N = 8) and in the active sTMS treated group (N = 8) (Fig.55A), 30 days post-daily sTMS treatment. In the active sTMS treatment (2x 1.1 T) this threshold was significantly raised (median: 400 µC, IQ: 200 – 700 µC; U = 15.5, P = 0.021, r = -0.585; Fig. 55B), compared to the sham control group (median: 800 µC, IQ: 800 – 1100 µC).

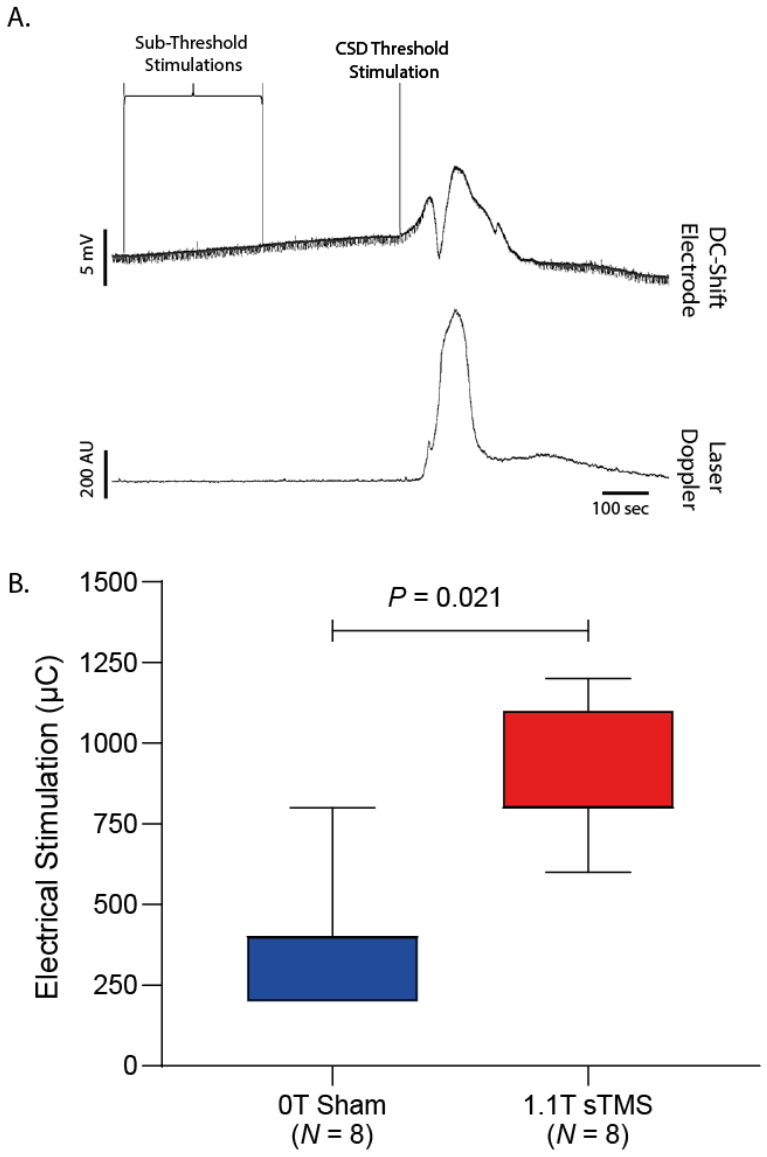

***Figure 55**: **Daily 1.1 T sTMS application increases electrical threshold for CSD initiation** (A) Example of an electrically evoked CSD wave in an animal treated with sham sTMS. Subthreshold stimulation with 400 µC and 600 µC ; at 800 µC a wave of CSD is evoked. (B) 30 days Daily sTMS at ~1.1 T significantly increased the electrical threshold required to generate a CSD compared to the sham stimulation group (0 T). (P = 0.021; Mann-Whitney test)*

Actions of long-term sTMS treatment on electrical activation threshold of third order VPM neurons

The electrical stimulation threshold for third order neurons of the VPM thalamic nucleus was determined in the daily sham-treated group (N = 5, n = 75; median: 16.0 V, IQ: 13.8 – 21.9 V; Fig. 56A) and active sTMS treated group (N = 5; median: 26.8 V, IQ: 24.9 – 29.4 V). In the active sTMS treatment (4 x 1.1 T daily) activation threshold was significantly raised (U = 0.00, P = 0.009, r = -0.826; Fig. 56B).

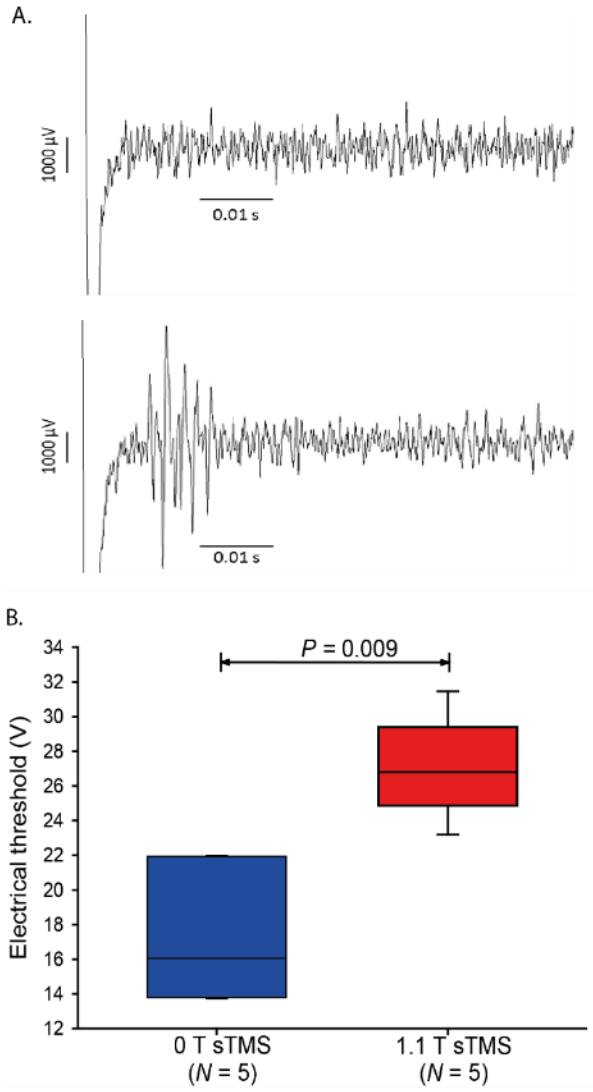

Figure 56: Daily 1.1 T sTMS application increases electrical activation threshold for 3rd order thalamic neurons *(A) Example of a post-SSS stimulation trace from third order neurones showing no evoked action potentials with 12 V sub-threshold stimulation and evoked action potentials with 14 V stimulation of the SSS. (B) Thirty days of daily sTMS treatment at ~1.1 T significantly increased the electrical threshold required to induce evoked action potentials in response to trigeminovascular stimulation in third order thalamic neurons compared to the sham stimulation group (0 T). (P = 0.009; Mann-Whitney test)*

Actions of long-term sTMS treatment on CSD-evoked changes on spontaneous activity of third order neurons

At the end of the 30 days sTMS treatment period, third order neurons in the VPM of animals in the active and sham treatment group were tested for their response to a pinprick induced CSD. In the sham treated group, the spontaneous activity of third order neurons showed a large reduction for 10 minutes post-CSD in all cells recorded (N = 4, 57.27 ± 5.25 % of baseline firing, Fig. 57A). A reduction from baseline was also seen in a subset of recorded cells in the 1.1 T sTMS group (N = 3, 46.37 ± 5.80 % of baseline firing), while a second subset of recorded cells showed facilitation of spontaneous neuronal activity following CSD induction (N = 2, 140.57 ± 11.94 % of baseline firing, Fig. 57B). However, the number or cells recorded were not large enough to carry out any reliable statistical comparison of the groups.

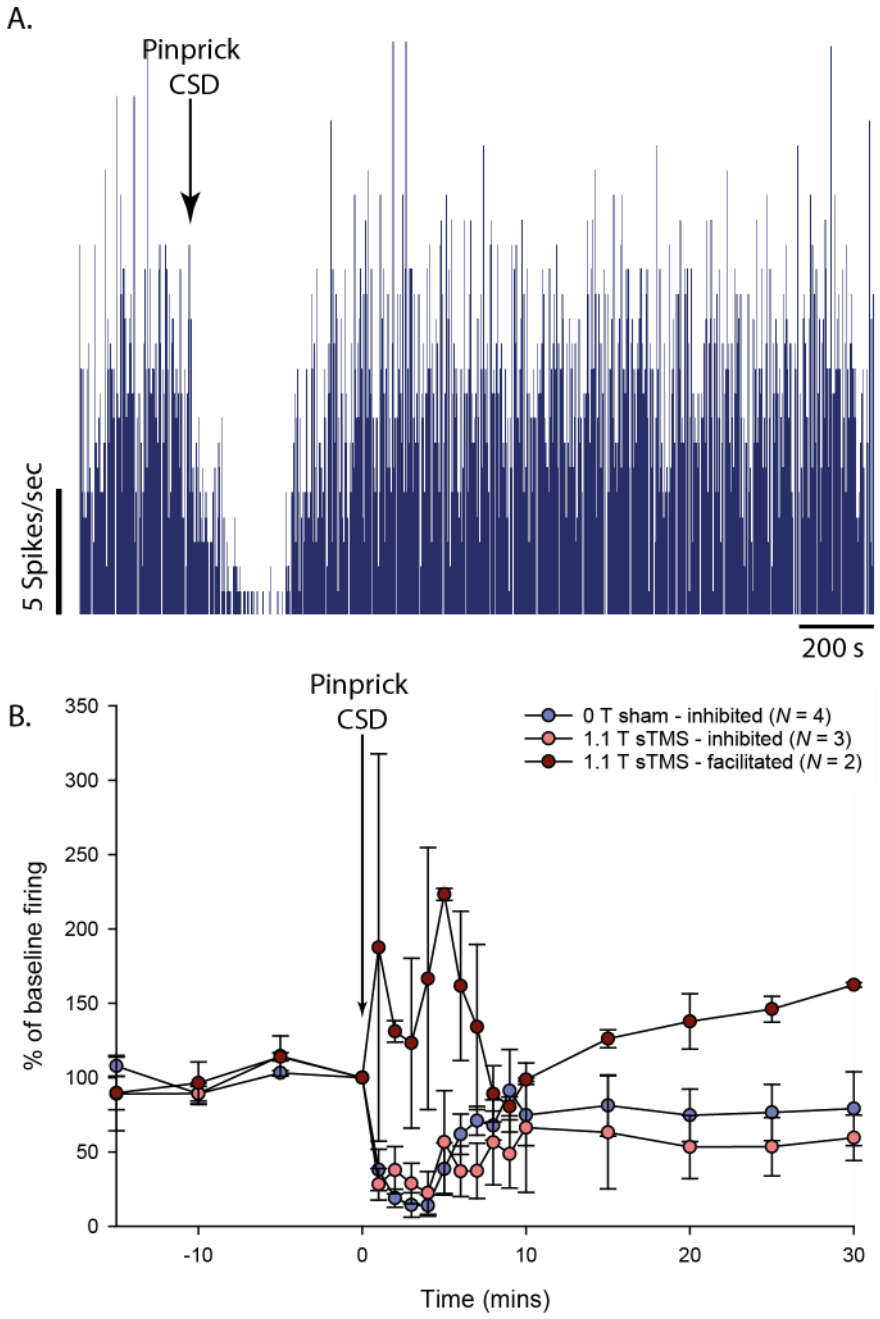

Figure 57: The effect of CSD on 3rd order neurons could not be reliably determined *(A) Example of a spontaneous thalamic neuronal activity of a third order VPM neuron in an animal treated with sham sTMS during a CSD. (B) Inhibition of spontaneous activity was seen in both 0 T sham and 1.1 T sTMS application groups. A subset of 1.1 T sTMS group also showed facilitation of spontaneous activity following CSD application, while the remaining cells showed inhibition following CSD induction. Number of experiments were not powered enough to carry proper statistical analysis.*

Discussion

The data shown here demonstrated an increase in the thresholds for CSD initiation (in the cortex) and third order neurons (in the thalamus), similar to what was shown previously with acute application of 1.1 T sTMS in chapters 3 and chapter 4 (respectively), suggesting a cumulative inhibition of excitability by sTMS. However, there was no change in activation of second order neurons compared with 0 T sham stimulation. This could suggest that long-term sTMS' effect is limited to its direct actions on the cortex, and indirect actions on subcortical nuclei, such as the thalamus. In addition, in line with the current clinical knowledge on the preventive use of sTMS, long-term use of sTMS does not appear to sensitise the trigeminovascular system.

Does long-term application of sTMS induce "Machine overuse headache"?

One of the main side effects reported from patients receiving sTMS as a preventative treatment for migraine is a headache and sensitivity at the site of application (Starling et al., 2018). This might suggest that sTMS activates peripheral trigeminal neurons that could lead to the development of headache, perhaps similar to MOH. In the trigeminovascular experiments performed here following long-term application of sTMS, the lack of FOS activation in TCC neurons seen here, would suggest that long-term application of sTMS does not induce sensitisation of second order neurons, that is normally expected to be seen in MOH models (De Felice et al., 2010). This also corroborates previous research by Andreou et al. who found that acute application of sTMS did not alter firing rates of second order neurons in the TCC (Andreou et al., 2016).

The behavioural experiments performed here, did not allow us to reach any conclusion. Behavioural responses to von Frey stimulation of both the 0 T sham and 1.1 T sTMS groups, showed no additional peri-orbital mechanical sensitisation. There was no significant difference between the sham and sTMS groups, perhaps suggesting that sTMS does not have a direct stimulating impact on cephalic and dural trigeminal fibres that could induce their sensitization. However, both groups did show a reduction from baseline, potentially masking any sensitising effect in the treatment group, further explanation of this will be discussed below. Overall, there is no evidence to suggest that long-term sTMS application induces "machine overuse headache".

Chapter 4:
Long term application of sTMS

Mechanism of action of sTMS as a preventive treatment

In previous chapters I have been able to show that acute application of sTMS is able to increase the threshold for CSD initiation and thalamic activation. The similar increase in the threshold for CSD initiation and thalamic activation, shown here after long-term sTMS application, suggests a cumulative inhibition of both the cortex and thalamus beyond the acute effects of sTMS.

Hyperexcitability of the cortex is thought to be a driving factor in the susceptibility to CSD (and thus aura) in migraine patients (Chronicle et al., 2006). Additionally, cortical hyperexcitability may also contribute towards the sensitisation of the trigeminal ganglion (Bolay et al., 2002), and chronification of migraine (Aurora et al., 2007). In chapter 3 (acute direct effects of sTMS application on cortical neuronal activity) I was able to show that sTMS' cortical effect (increasing CSD threshold and inhibiting glutamate induced activity) appears to be mediated by interaction with GABAergic systems, which corroborates previous research (Murphy et al., 2016). During long-term sTMS application, a similar mechanism may underlie the cumulative inhibition of cortical excitability seen and would be worth investigating in the future. It would be interesting to investigate if concurrent blocking of the cortical GABAergic system would negate the cumulative long-term effect of sTMS. Unfortunately, the GABA antagonists, such as bicuculline and saclofen (as were used in chapter 3) cannot be applied globally or over a prolonged period to the cortex as they are proconvulsant (Treiman, 2001). Alternatively, action of long-term application of sTMS on GABAergic system could be investigated using an intensity-based GABA sensing fluorescence reporter (iGABASnFR) mouse model (Marvin et al., 2019) and *in vivo* calcium imaging to directly record cortical GABA activity. Immunohistochemistry could also be used to gain a snapshot of expression of proteins associated with cortical GABA: synthesis ($GAD_{65/67}$), receptors ($GABA_{A/B}$) or metabolites (GHB), following 30 days of daily sTMS treatment.

The thalamus acts as a relay for ascending somatosensory information, by reducing ascending thalamic excitability (as seen by the increase in activation thresholds following trigeminovascular stimulation) long-term sTMS could prevent the further processing of hyperalgesic trigeminal signals. In the previous chapter it was suggested that the inhibition of thalamic activity was driven by a switch from high to low frequency firing

by descending cortico-thalamic afferents, recruiting inhibitory thalamic reticular formation modulation of the thalamus. The increase in the threshold of trigeminovascular evoked thalamic activity caused by chronic application of sTMS (+ 9.56 V ± 1.18) was of a similar extent as during the acute treatment (+ 6.75 V ± 3.02), therefore a similar mechanism may underlie both the acute and cumulative inhibition of thalamic excitability.

Following CSD, a similar degree of inhibition was seen in thalamic neurons in animals that had received long-term sTMS application as the 0 T sham. Two further animals, in the sTMS group, demonstrated facilitation of thalamic activity following CSD. Because of the difference in response to CSD the N numbers were not significant to perform statistical analysis, therefore no conclusion can be drawn from the data. Time restraints precluded further investigation. Both sets of thalamic experiments were carried out in succession on the same animals, in an effort to reduce animal numbers as per 3Rs recommendations (Prescott and Lidster, 2017).

Limitations

The main limitation to the behavioural mechanical pain testing is the lack of a negative control, i.e., animals that received no positioning of the sTMS coil over their head daily. The 0 T sham group also saw a drop in periorbital response thresholds in both sets of experiments. It became clear throughout the experiment that animals were stressed from both the TMS applications and the periorbital pain testing. In both instances as the testing period progressed, it became obvious that animals learnt to shield their heads underneath themselves or in the corner of the von Frey box. Unfortunately, with the current setup, it was not possible to administer the sTMS without removing animals from their home cage, separating them and handling them daily, all of which can cause stress (Prescott and Lidster, 2017). This stress from the daily treatment is believed to be the main cause of the altered responses to von Frey stimulation and pain thresholds.

Any future experiments could further improve the mechanical pain behaviour findings. A longer pre-treatment training period could further familiarise the animals with the setup, improving the stress response (Hurst and West, 2010). The suggestion was made to lightly

anaesthetise the animals with isoflurane during sTMS application, however this introduces an additional confounding variable to the experiment. Although the precise mechanism of action of isoflurane remains unclear, isoflurane has been linked to $GABA_A$ receptors of which we have previously shown sTMS interacts with. One of the advantages of this set of experiments was to observe the effects of sTMS on awake unanaesthetised animals. The ideal setup would be to be able to apply sTMS to the animals in their home cages on a set schedule without the experimenters even being present. However, with the current setup this is not possible. Additional behavioural tests could be run on the animals to try and isolate out stress behaviours from nociceptive behaviours, however this would mean increased daily interaction with the animals which may only exacerbate the issue of stress.

The behavioural testing and treatment was carried out by the same male individual. To try to maintain blinding, behavioural testing was carried out first, with the treatment groups hidden, followed by unblinding and daily TMS treatment. Every effort was made to remain blind to the treatment group during the testing phase to minimise any experimenter influences on the results, however it is unavoidable that familiarity with the animals as the experiment progresses could introduce some bias to the interpretation of animal behaviours. In future experiments it would be beneficial to have separate investigators applying the TMS and performing behavioural tests. This would improve blinding, removing any possibility of bias and could additionally help to reduce stress in the animals during behavioural testing. Having separate investigators would also mean the acute effects of sTMS on animal behaviour immediately following application could be investigated. Although some unblinding was unavoidable, data presented here suggest that bias was not an issue in this study.

The investigator carrying out the behavioural testing was male. Olfactory exposure to males has been shown to induce stress in animal models, altering plasma corticosterone levels and consequently altering behavioural responses (Sorge et al., 2014). While in the short-term increased cortisol has an analgesic effect, chronically increased levels have been shown to enhance nociception (Gamaro et al., 1998, Metz et al., 2001, Costa et al., 2005). Minimising male exposure (by having a female investigator carry out the

behavioural testing) may help to minimise this stress response, which may be masking any subtle effects long-term sTMS application may be producing.

In humans, the development of MOH is dependent on both the overuse of analgesics but also a pre-existing headache disorder. These experiments were carried out on naïve animals without any predisposition to developing headache attacks, thus just applying the sTMS may not be enough to drive an analgesic overuse headache. Previous research by de Felice et al. were able to show triptan-induced sensitisation in naïve animals and suggested an animal model of MOH could be produced in naïve animals (De Felice et al., 2010). An animal model with pre-exposure to headache inducing agents, such as nitro-glycerine (Greco et al., 2018, Tassorelli et al., 2003) could be utilised, to investigate the actions of sTMS on a sensitised system.

The main limitation of the electrophysiological experiments that followed the 30-day application of sTMS, was the limited number of animals. In the thalamic response to CSD experiments particularly, low animal numbers prevented reliable statistical comparison between the 0 T sham and 1.1 T sTMS groups. The main obstacle for carrying out further experiments, was the considerable investment of time and resources required. In general, each individual electrophysiological experiment takes full day to complete (with the exception of CSD threshold experiments that could be carried out 2 per day), thus an additional 1-2 weeks of experimental time was required on top of the daily 30-day sTMS application. With 4 separate sets of 30-day trials (plus additional time for post-trial testing) a total of ~6 months was spent investigating the long-term application of sTMS.

Conclusion

In conclusion, this series of experiments demonstrated that treatment with sTMS appears to have a long-lasting effect beyond the acute actions shown previously. Daily application of sTMS alters the excitability of the directly affected cortex and indirectly affected cortico-thalamic networks. Such changes are not observed on second order neurons. Potentially this is due to the lack of a vast cortico-spinal network as opposed to the massive corticothalamic modulatory pathway. Additionally, this suggests that sTMS does not have a direct stimulating impact on second order neurons (and potentially also cephalic and dural trigeminal fibres) that could induce their sensitization.

Chapter 5: sTMS clinical audit

Chapter 5:

sTMS clinical audit

Introduction

Migraine is a common and often disabling neurological condition (Vos et al., 2017). Per year in the UK, it is estimated that the National Health Service (NHS) spends approximately £150 million on migraine patients with a further £100 million on wider headache conditions, primarily from appointments and treatments (Stephen O'Brien et al., 2010). Around 3% of the general population will consult primary care for headache disorders each year amounting to ~2.5 million primary health care appointments, ~4% of all general practitioners' (GP) visits each year (Latinovic et al., 2006). From initial GP visits, ~100,000 referrals are made to hospitals concerning headache disorders, accounting for ~33% of neurology referrals (Latinovic et al., 2006). Tertiary healthcare centres can provide highly specialised services for specific disorders and require referral from secondary healthcare providers. Across the UK there are approximately 50 clinics specialising in headache disorders (Trust, 2019).

In patients with frequent migraine symptoms, pharmacological treatments constitute the main preventive strategy. However, the established migraine pharmacotherapy is often associated with efficacy, tolerability and adherence issues (Hepp et al., 2014, Rahimtoola et al., 2003). Chronic migraine (CM) more than episodic migraine (EM) patients discontinue/switch between treatments largely because of lack of efficacy and/or tolerability issues (Ford et al., 2017). Moreover, only a small proportion of CM patients adhere long term to pharmacological treatments over a period of one year (Hepp et al., 2017). Adherence and tolerability issues may be mitigated with the introduction of the novel monoclonal antibodies anti-calcitonin gene related peptide (CGRP), which seem to have a better tolerability profile compared to oral tablets. However clinical trials data show a response rate of about 40 - 50% in CM patients (Dodick et al., 2018a), highlighting the still unresolved unmet need in migraine therapy.

Non-invasive neuromodulation approaches have emerged as an alternative to pharmacological treatments in headache treatments (Lambru and Lanteri-Minet, 2019). The rationale of these treatments is to improve headache and associated symptoms by altering the neural tissue activity of pathophysiological relevant targets in a non-invasive fashion. One of the most promising of such treatments includes single pulse transcranial

magnetic stimulation (sTMS). In 2010 Lipton et al. carried out a randomised, double-blind, parallel-group, two-phase, sham-controlled, multi-centre study. Eighty-two migraine with aura patients treated at least one migraine attack with sTMS and were compared against a further 82 migraine with aura patients treated with a sham stimulation. Pain freedom 2 hours after treatment showed a 17% therapeutic gain in the sTMS vs the sham treatment. This appeared to be a sustained effect with greater proportion of the sTMS group remaining pain free 24 and 48 hours post treatment. There were no reported serious adverse events, with the remaining reported adverse events (Headache, Migraine, sinusitis and paraesthesia) not significantly different between the two treatment groups (Lipton et al., 2010). Following FDA approval, an open-label study testing the efficacy of three months sTMS treatment in episodic migraine, suggested that almost half of patients obtained at least a 50% reduction in headache days (Starling et al., 2018). Additionally, a company sponsored UK-based post market audit reviewed the effect of both acute and continuous use of sTMS device for three months in 449 patients with predominantly chronic migraine. Although the audit showed good short-term tolerability and efficacy, the analysis was conducted only in 190 of the 449 patients (42%), suggesting caution in data interpretation (Bhola et al., 2015).

In view of these evidence, Spring sTMS is CE-marked in Europe and obtained National Institute for Health and Care Excellence (NICE) UK approval in 2014, although without a technology appraisal guidance. Practically this did not allow for funding in order for NHS to use this treatment. Another limitation of the NICE approval is that it does not appropriately distinguish between rTMS and sTMS; NICE approval is for TMS. As previously discussed, no established protocol for rTMS treatment in migraine has been trailed properly. NICE recommendation includes the conduction of clinical audits that will re-enforce the available data on the efficacy and safety of TMS (Table 8).

The Headache centre at Guy's and St Thomas' NHS Foundation Trust (GSTT) is a tertiary clinic in central London, specialising in headache and facial pain disorders. It is the only UK Headache Centre that, in 2017, obtained commissioners' funding to provide the sTMS treatment as a free-of-charge treatment within the NHS England, without the need to apply for individual patient funding. The clinic has since prescribed the sTMS

treatment to a number of migraine patients, allowing the performance of a clinical audit for this treatment.

Clinical audits are defined by NICE as;

> *"A quality improvement process that seeks to improve patient care and outcomes through systematic review of care against explicit criteria and the implementation of change."* (Scrivener et al., 2002)

Where research aims to develop a program of best clinical practice, clinical audits aim to evaluate how closely the currently delivered treatment is to that clinical practice. NICE suggests three criteria for the clinical audit of transcranial magnetic stimulation for treating and preventing migraine;

1) The percentage of patients undergoing transcranial magnetic stimulation for treating and preventing migraine who have had any of the following clinical outcomes: improvement in headache, reduction in the number of migraine attacks, days, hours, pain intensity, reduction in the use of analgesics for acute attacks, reduction in the use of prophylactic medication and improvement in quality of life
2) The percentage of patients undergoing transcranial magnetic stimulation for treating and preventing migraine who have had any of the following adverse events: dizziness or discomfort.
3) The percentage of patients undergoing transcranial magnetic stimulation for treating and preventing migraine who have: been told that there are uncertainties about the procedure's safety and efficacy, received written information explaining that there are uncertainties about the procedure's safety and efficacy, and given written consent to treatment (full details in Table 8) (NICE, 2014a).

Research in sTMS therapy lacks independent, long-term efficacy and safety data in difficult to-treat CM migraine patients who have already failed pharmacological approaches.

For this reason, we conducted a prospective clinical audit on the NHS to evaluate the long-term effectiveness safety and tolerability of sTMS in migraine, according to the NICE UK audit framework (NICE, 2014a).

Table 8: Audit criteria from Clinical audit tool: Transcranial magnetic stimulation for treating and preventing migraine (NICE, 2014a)

Criterion 1	The percentage of patients undergoing transcranial magnetic stimulation for treating and preventing migraine who have had any of the following clinical outcomes: • pain-free response for the first treated attack • improvement in headache • reduction in the number of migraine attacks, days, hours, pain intensity • reduction in the use of analgesics for acute attacks • reduction in the use of prophylactic medication • improvement in quality of life • other.
Exceptions	None
Standard	Outcomes from published literature should be considered when reviewing audit data, such as those set out in the guidance
Definitions	Quality of life could be measured using the MIDAS and HIT-6 scores.
Criterion 2	The percentage of patients undergoing transcranial magnetic stimulation for treating and preventing migraine who have had any of the following adverse events: • dizziness • discomfort other.
Exceptions	• None
Standard	Adverse events from published literature should be considered when reviewing audit data, such as those set out in the guidance
Definitions	**Adverse-event grades** 0: No adverse event I: Any deviation from the normal postoperative course without the need for pharmacological treatment or surgical, endoscopic and radiological interventions

	II: Requiring pharmacological treatment with drugs other than such allowed for grade-I complications. Blood transfusions and local parenteral nutrition are also included III: Requiring surgical, endoscopic or radiological intervention IIIa: Intervention not under general anaesthesia IIIb: Intervention under general anaesthesia IV: Life-threatening complication (including central nervous system complications) requiring intermediate care/intensive care unit management IVa: Single organ dysfunction (including dialysis) IVb: Multi-organ dysfunction V: Death of a patient Suffix 'd': If the patient suffers from a complication at the same time of discharge, the suffix 'd' (for 'disability') is added to the respective grade of complication. This label indicates the need for a follow-up to fully evaluate the complication For further definition of these grades please visit www.surgicalcomplication.info
Criterion 3	The percentage of patients undergoing transcranial magnetic stimulation for treating and preventing migraine who have: • been told that there are uncertainties about the procedure's safety and efficacy • received written information explaining that there are uncertainties about the procedure's safety and efficacy • given written consent to treatment.
Exceptions	If the patient is unable to understand information and/or give consent to treatment.
Standard	100%
Definitions	NICE recommends its Information for the public. This document is written to help patients who have been offered this procedure (and their families or carers) to decide whether to agree to it or not.

Chapter 5:
sTMS clinical audit

Methods
This audit was part of a service evaluation conducted at the Headache Service at Guy's and St Thomas' NHS Foundation Trust, London, UK. New patients were included in the audit between January 2017 and July 2020. Audit under current national guidelines does not require research ethics committee review (MRC and HRA, 2017)

Participants
Consecutive adult patients attending the Headache Service and meeting the International Headache Society (IHS) criteria for CM or for episodic migraine, with at least eight migraine days/month (high frequency) (IHS, 2018) who failed at least three preventive treatments, were included in the audit. Treatment failure was defined as treatment discontinuation due to unacceptable side effects and/or absence of reduction in headache frequency, duration or severity after administration of a preventive medication for at least 12 weeks. Contraindicated treatments were not considered as treatment failures. For patients who underwent a trial with botulinum toxin type A (BoNT/A), failure to obtain at least 30% reduction in headache days after two sets of injections three months apart was considered treatment failure as per NICE UK guidance (NICE, 2019b). Patients with MOH were not excluded from the audit. When MOH was present, withdrawal attempts using outpatients pharmacological and non-pharmacological strategies were tried. Patients could continue oral preventive medications during treatment with sTMS, although we advised not to change the medications dose during the first three-month trial to avoid confounding the outcomes. Patients had to be compliant with device use, daily headache diary and 3-monthly headache impact test (HIT-6) completion to be part of this analysis. Patients with a personal history of epilepsy and/or implanted devices were excluded.

Device use and treatment protocol
Patients were demonstrated how to use the sTMS device by our trained headache nurses (B.H., M.M.) (see Table 9 for device technical specifications). Once turned on the sTMS device takes approximately 30 - 60 seconds for the capacitors to reach full charge,

indicated by the LED progress bar surrounding the power button. Once at full charge the device can be positioned at the base of the skull (Fig. 58A), a 0.9 T pulse (measured 1 cm from the surface) was delivered within 45 seconds, by pressing and holding both the trigger buttons on either side of the device for at least 2 seconds. As the treatment is delivered the device produces an audible click, some patients have additionally described a tactile sensation. Pressing the power button recharged the device, for further treatments, otherwise the device shuts off after 10 seconds. Repeated applications caused the devices surface to heat up to temperatures in excess of 40°c, in which case it was advised to allow the device to cool for 15 minutes. Patients were encouraged to use the device prophylactically following a titration protocol as well as to abort migraine attacks when needed (Table 10).

Table 9: Spring TMS Device Technical Specifications

Weight	1.4 kg
Size	Length: 22.4 cm, width: 13 cm, depth: 6.9 cm
Mode of Operation	Short-time operation – individual 1ms pulses
Magnetic Pulse Output	0.9 Tesla (nominal) at 1 cm from the centre of curved surface of the sTMS mini per treatment
Current	4 mA/cm^2 induces at 1 cm
Pulse Duration	180 µs (total magnetic energy 140 J)
Service Life	1800 treatments (sTMS mini device, AC Adapter and Rx SIM Card)
Operating environment	Temperature range: 15°C to 40°C; Humidity range: 10% to 90%, non-condensing; Atmospheric pressure range: 700 hPa to 1013 hPA; Operating altitude: less than or equal to 3000 m
Electrical Power	Internally powered (Rechargeable battery)
Rechargeable Battery	7.2 V, 2150 mAh, 15.48 Wh lithium-ion battery Typical battery life is 100 charge cycles (minimum)

Table 10: Single-pulse transcranial magnetic stimulation device treatment protocol

Preventive treatment titration protocol	
Week 1	Deliver 2 sequential pulses twice daily
Week 3	Deliver 2 sequential pulses three times daily
Week 5	Deliver 3 sequential pulses three times daily
Week 7	Deliver 4 sequential pulses three times daily
Week 9	Deliver 5 sequential pulses three times daily
Week 11	Deliver 6 sequential pulses three times daily
Abortive treatment protocol	Deliver as early as possible 2 sequential pulses every 15 minutes for 1-2 hours or until pain and symptoms resolve.

Outcome measures

Details of the audit timeline are shown in Figure 58B. A migraine-specific diary and the Headache Impact Test-6 (HIT-6) score were used to capture effectiveness and disability measures. Patients were required to produce a baseline headache diary and HIT-6 score for at least one month prior to treatment initiation and to continue filling the headache diary on a daily basis along with HIT-6 scores following the conclusion of the 3-month trial. Data were entered in an electronic macro database for analysis.

The cut-off outcome for treatment continuation was reduction in the mean monthly headache days (MHD) of at least 30% after three months of treatment (headache frequency responders). Additionally, patients who experienced a ≥5-point reduction in the HIT-6 even if they did not meet the 30% mean MHD reduction were offered treatment continuation (headache disability responders). Other effectiveness outcomes analysed at month 3 included: changes from baseline in the MHD and in the monthly migraine days (MMD), proportion of patient with at least 50% reduction in MHD, change in monthly headache-free days and change in monthly abortive treatment intake use days. The same effectiveness treatment outcomes were re-assessed in those who continued the treatment

for at least 12 months. A “headache day” was defined as a day with headache lasting for ≥4 hours and with a severity of ≥4/10 on a verbal rating scale (0, no head pain, 10 worst pain ever experienced). A “migraine day” was defined according to the IHS classification criteria (2018), as a “headache day” with additional associated symptoms (nausea, vomiting, photophobia, phonophobia or motion sensitivity, or use of an abortive triptan. A “headache-free day” was defined as a day without any head pain. Changes in abortive treatment intake days and change in the proportion of patients with MOH were also evaluated. An “abortive treatment intake day” was considered any day where patients consumed abortive treatments for attempted headache relief. To assess whether any change in effectiveness measures was associated with improvement in headache-related disability, change in HIT-6 score was analysed.

Subgroup analysis of the primary and secondary efficacy outcome measures were carried out between patients who, at baseline, presented with migraine with and without aura, migraine with and without medication overuse headache (MOH) and BoNT/A responders versus non-responders and patients presenting with and without vestibular symptoms.

Patients were asked about the development of adverse events (AEs) during telephone follow-ups or clinical appointments for the subsequent months. Adverse events were graded as mild, moderate and severe.

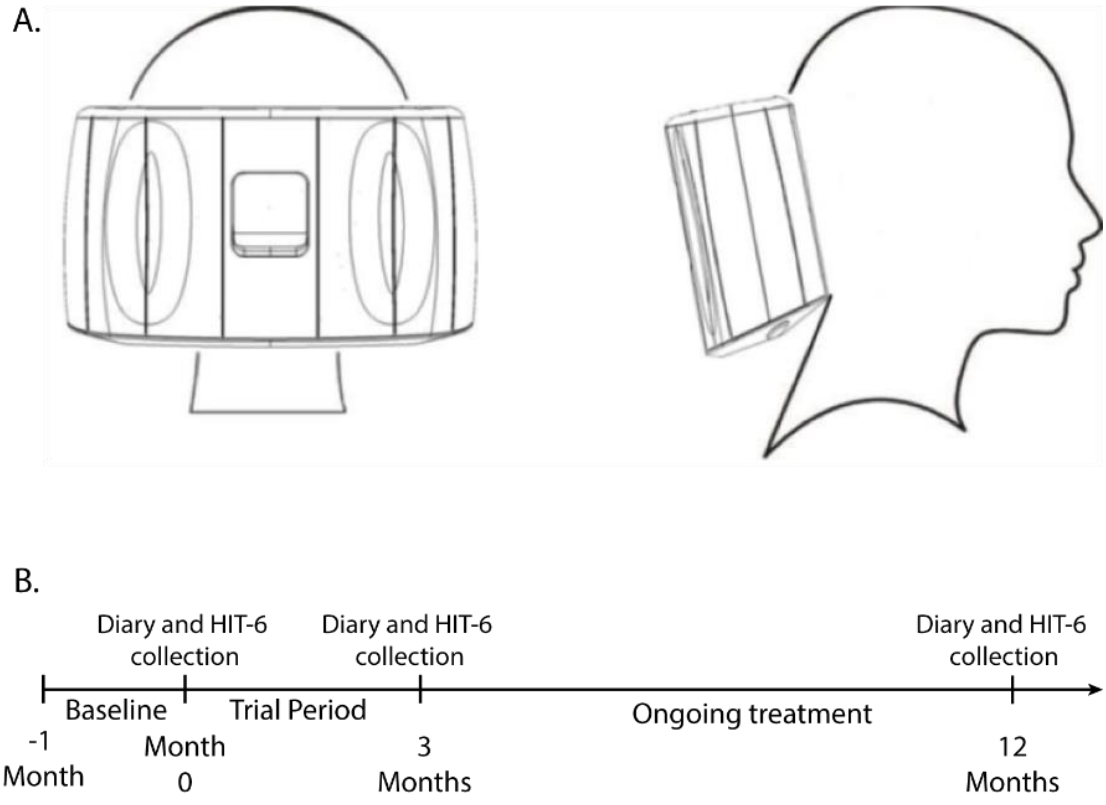

Figure 58: sTMS preventative treatment setup *(A) Spring TMS optimal placement and coil locations (B) Diagram illustrating the clinical audit timeline*

Statistical analysis

All outcomes pre- and post-sTMS treatment were measured on a continuous scale. For all measures considered here, data demonstrated a skewed distribution with a significant deviation from normal distribution (Kolmogorov-Smirnov test; $P < 0.05$). As a result, the Wilcoxon signed ranks test was used to compare the change in values over time. For independent group comparison the Mann-Whitney test was used. The Z value of these tests was used to calculate the effect size r, as Z statistic divided by the square root of the sample size (N):

$$r = \frac{Z}{\sqrt{N}}$$

P-values of less than 0.05 were regarded as evidence of a statistically significant result. Effects of sTMS between and within groups were analysed using SPSS statistics 23 (IBM, USA). We identified nine missing diaries at month 12 from nine patients. Any missing values were treated in SPSS as discrete missing values. All data are provided as median

(interquartile range (IQR)), unless stated otherwise. Where relevant, patient numbers have additionally been given as a percentage of all registered patients as a intention to treat (ITT) analysis.

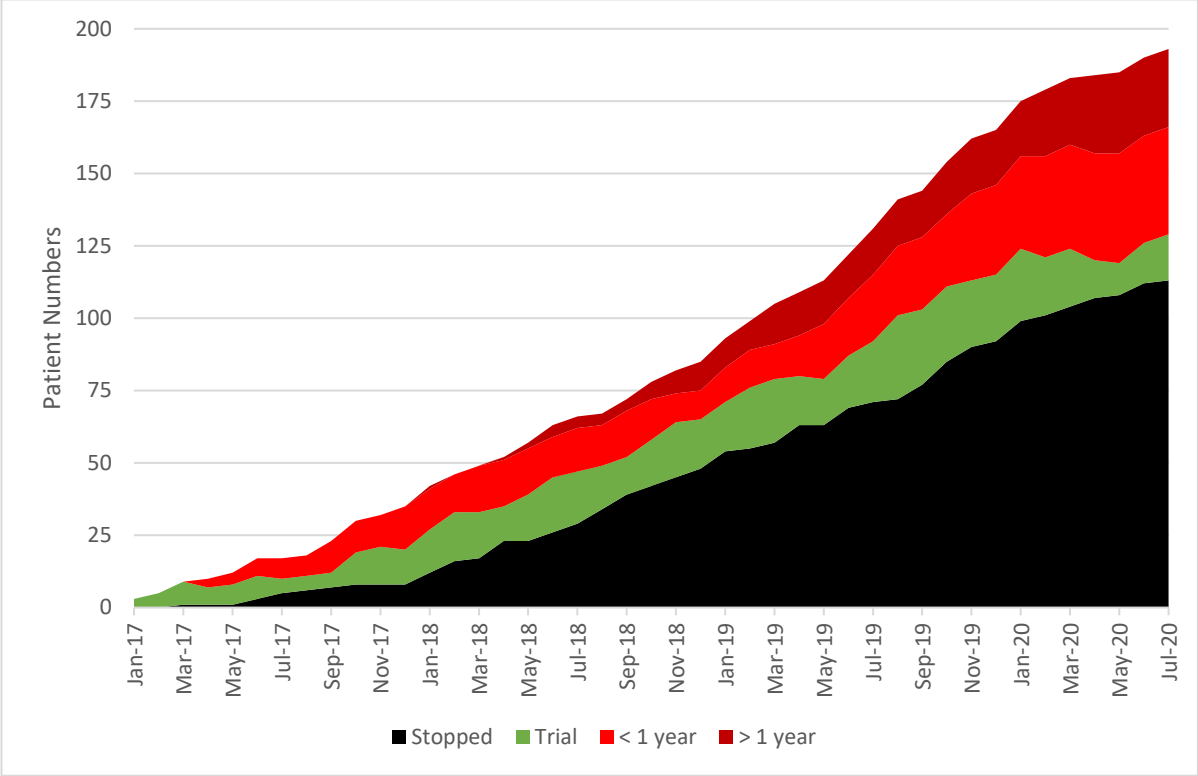

Figure 59: Patient number growth at GSTT sTMS clinic as of July 2020

Chapter 5:
sTMS clinical audit

Results

Demographic and baseline headache characteristics

The sTMS service began in January 2017. Since then, a total of 214 patients have been prescribed the therapy [176 female; median age: 44.0 years IQR: 34.0 – 58.0] (figure 59). At the time of the analysis, 153 patients completed the 3-month treatment trial and hence were included in the audit. Completed headache diaries and HIT-6 for all months during the 3-month trial period, were obtained by 153 patients who were included in the analysis. 16 patients failed to provide Completed headache diaries and HIT-6 and therefore were excluded from the audit. 43 patients /started treatment after 1st August 2019 and therefore were excluded from the audit due to complications arising from the ongoing COVID-19 pandemic (Figure 60). Demographic and clinical characteristics of the patients' group at baseline are summarised in Table 11. To qualify for inclusion in the clinic, all patients had failed at least three oral preventive treatments, of whom 72.5% (111/153) (ITT: 52%, 111/214) also failed BoNT/A before trialling sTMS. Most patients (93.0 %) were classified in the severe impact category at baseline (HIT-6 score: 60-78).

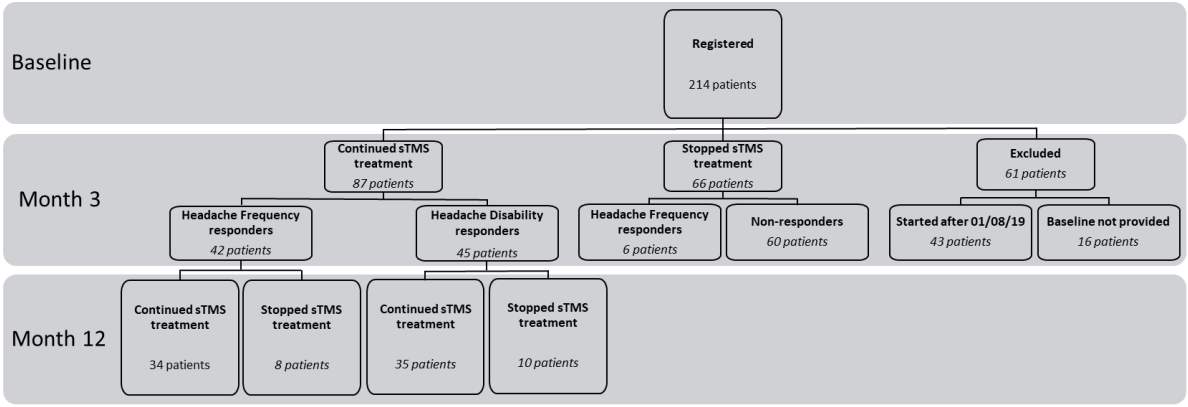

Figure 60: Audit design Of the 153 patients analysed, 48 patients achieved at least a 30% reduction in mean monthly headache days at month 3. Of those, 32 patients achieved more than 50% reduction in mean migraine days, while four patients who achieved at least a 30% reduction decided to stop the treatment due to adverse events. Of the remaining patients who did not achieve a 30% reduction in mean monthly headache days, 60 discontinued the treatment, while 45 wished to continue the sTMS treatment. Overall, at month 3, 57% of patients who were initially prescribed the treatment, continued using sTMS. At 12 months, 8 out of the 42 patients who achieved at least a 30% reduction at month 3, stopped the treatment due to reduced or inconsistent efficacy. Of the 45 patients who chose to continue the treatment without achieving a 30% reduction at month 3, 35 of them continued using the sTMS treatment at 12 months, whereas the remaining 10 discontinued. Overall, by month 12 45% of patients who were initially prescribed sTMS continued using the treatment.

Table 11: Demographic and clinical characteristics at baseline of all migraine patients treated with single pulse transcranial magnetic stimulation

	Total number of patients
Sex, M/F	38/176
Age (y), Median (IQR)	44.0 (34.0 – 58.0)
Diagnosis, CM/HFEM/other	154/30/30
CM duration (y), Median (IQR)	13.5 (6.0 – 23.5)
Aura, *N* (%)	89 (58.1 %) (ITT: 41.6%)
Medication overuse, *N* (%)	83 (54.2 %) (ITT: 38.8%)
BoNT/A non-responders, *N* (%)	111 (72.5 %) (ITT: 51.9%)
	Median (IQR)
Headache days	18.0 (12.0 - 26.0)
Migraine days	13.0 (8.75 - 22.0)
Headache free days	5.0 (0.0 - 13.0)
Abortive treatment intake days	9.0 (3.75 - 14.0)
HIT-6 score	66.0 (64.0 - 69.0)

BoNTA, onabotulinum toxin A; **IQR,** interquartile range; **CM**, chronic migraine; **F**, female; **HFEM,** high frequency episodic migraine; **HIT-6,** headache impact test-6; **M,** male**; N,** number; **y**, years

Efficacy outcomes at month 3 and treatment continuation

Overall, compared to baseline, the median reduction in MHD for all patients at month 3 was 5.0 days, from 18.0 (IQR: 12.0 - 26.0) to 13.0 days (IQR: 5.75 - 24.0); (P = 0.002, r = -0.29; Fig. 61A) and the reduction in median MMD was 4.0 days, from 13.0 (IQR: 8.75 - 22.0) to 9.0 (IQR: 4.0 - 15.25); (P = 0.002, r = -0.29, Fig. 61B). Treatment with sTMS led to a reduction in the percentage of patients with MOH, from 51.6 % at baseline (N = 79/153) (ITT: 37%, 79/214), to 18.9 % at month 3 (N = 29/153) (ITT: 14%, 29/214).

At month 3, 56.9% of patients (N = 87/153) (ITT: 41%, 87/214) were considered responders to sTMS; 31.4%, (N = 48/153) (ITT: 22%, 48/214) were "headache frequency responders" who obtained at least a 30% reduction in MHD (median MHD at baseline:

16.5, (IQR: 10.25 - 21.0); median MHD at month 3: 5 (IQR: 3.0 - 9.0); $P < 0.001$; r = -0.87). Twenty-nine percent (N = 45/153) (ITT: 21%, 45/214) were "headache disability responders", who reported clinically meaningful and significant improvement in the HIT-6 score compared to baseline, from 66 (IQR: 65.0 - 68.0) to 62 (IQR: 58.0 - 65.0); ($P < 0.001$, r = -0.67). Of the 48 "headache frequency responders", the majority (n = 32, 66 %) achieved at least a 50% reduction in MHD and three patients (6.25%) became completely headache-free.

A total percentage of 43.1% of patients (N = 66/153) (ITT: 31%, 66/214) discontinued the treatment after the 3-month treatment trial, in view of lack of: effectiveness (39.3%) or satisfaction with achieved effectiveness (N = 6, 4%; "headache frequency responders"). One patient stopped due to adverse events.

Efficacy outcomes at month 12

The monthly headache characteristics of all patients who continued treatment after the 3-month trial with sTMS (N = 87/153) (ITT: 41%, 87/214) are shown in Table 12. Of the 87 responders at month 3, 69 patients continued to respond and use the sTMS treatment at month 12.

Of these, 34 obtained at least a 30% reduction, 26 obtained at least 50% reduction in MHD, five of which were headache-free at month 12. By month 12 a further 18 patients discontinued the sTMS treatment due to lack of sustained effectiveness. Of these, the majority (N = 10/18, 55%) belonged to the group of "headache disability responders" rather than "headache frequency responders", the majority of whom continued to respond. Overall, of the initial 153 patients prescribed the sTMS treatment, 45.1% were responders at month 12, of all 214 registered patients 32.2% continued to respond at month 12.

In patients who continued the treatment at month 12 (N = 68), the reduction in MHD was sustained and remained significant compared to baseline (median MHD at month 12: 12.0, (IQR: 5.0 - 20.0), $P < 0.001$, r = -0.55). Similarly, the reduction of MMD was sustained significant compared to baseline (median MMD at month 12: 6.0 (IQR: 3.0 - 11.0), $P < 0.001$, r = -0.50. Treatment with sTMS significantly increased the number of

headache-free days from 5.0 days (IQR: 0.0 - 13.0) at baseline, to 11.5 (IQR: 0.0 - 22.0), P = 0.003, r = -0.39. The number of abortive treatment days was significantly reduced from 9.0 days (IQR: 3.75 - 14.0) at baseline to 4.0 days (IQR: 1.75 - 9.0) at month 12, P = 0.009, r = -0.39. Continuation of treatment with sTMS reduced the percentage of patients with MOH from 51.6% (N = 79/153) (ITT: 36.9% 79/214) at baseline to 19.0% (N = 29/153) (ITT: 13.6%, 29/214) at month 3, to 8.0% (N = 7/87) (ITT: 3.3%, 7/214) at month 12.

By month 12 further 18 patients discontinued sTMS treatment due to lack of consistent efficacy. Of these, the majority (55%, 10/18) belonged to the group of “Headache disability responders” rather than “Headache frequency responders”, the majority of whom continued to respond. Overall, of the initial 153 patients prescribed the sTMS, 45% (69/153) continued using the treatment past the first 12 months.

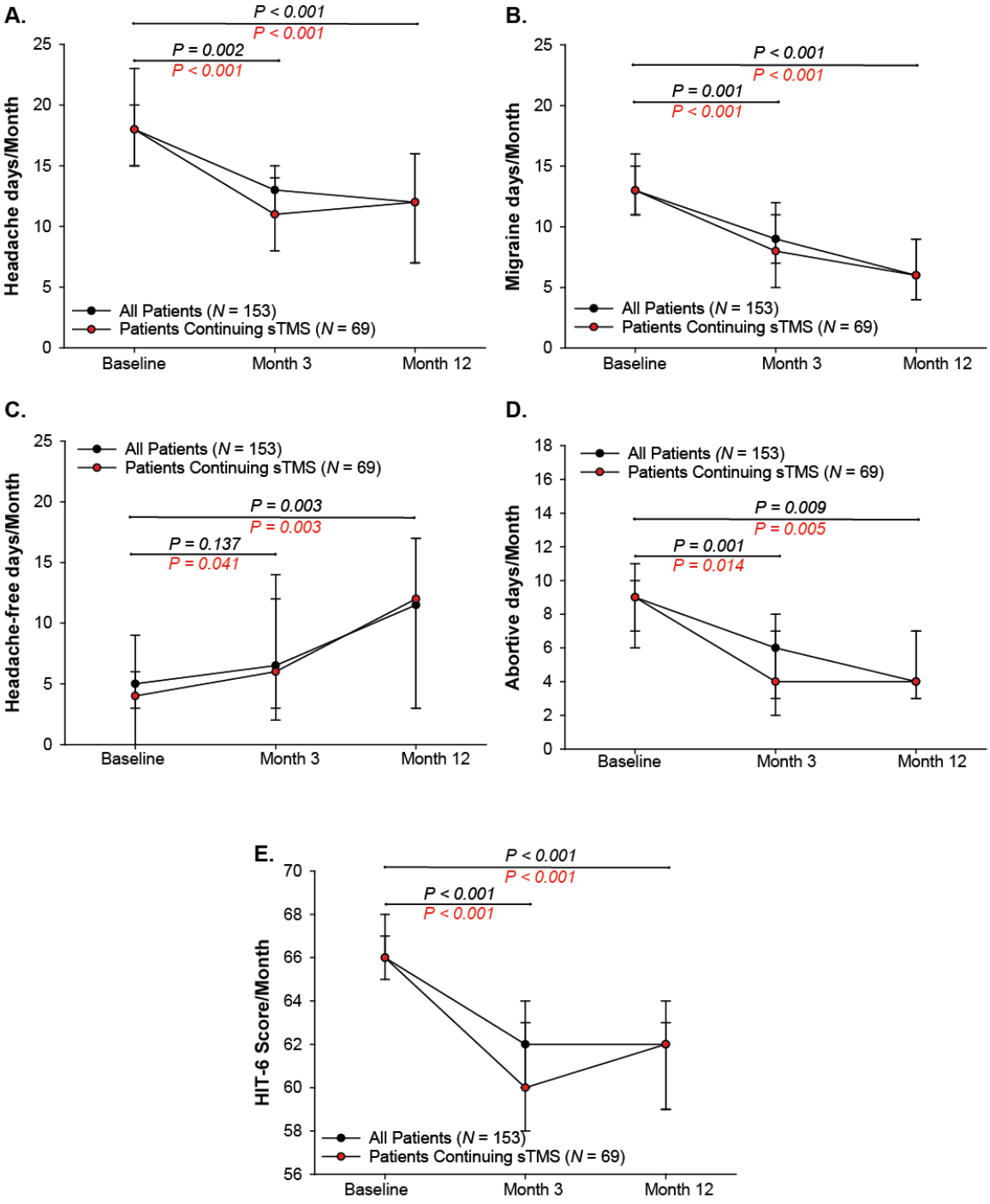

Figure 61: Clinical characteristics of all patients using single-pulse transcranial magnetic stimulation at baseline and at 3 and 12 months *(A) Mean headache days per month were significantly reduced from baseline at 3 (P = 0.002; Wilcoxon Signed ranks) and 12 months (P < 0.005; Wilcoxon Signed ranks) (B) Mean migraine days per month were significantly reduced from baseline at 3 (P = 0.001; Wilcoxon Signed ranks) and 12 months (P < 0.001; Wilcoxon Signed ranks) (C) Mean headache-free days per month were not significantly increased from baseline at 3 (P = 0.137; Wilcoxon Signed ranks) or at 12 months (P = 0.003; Wilcoxon Signed ranks) (D) Mean abortive days per month were not significantly increased from baseline at 3 (P = 0.001; Wilcoxon Signed ranks) but was at 12 months (P = 0.009; Wilcoxon Signed ranks) (E) Mean HIT-6 score was significantly reduced from baseline at 3 (P < 0.001; Wilcoxon Signed ranks) and 12 months (P < 0.001; Wilcoxon Signed ranks).*

Table 12: Clinical characteristics at baseline and at 3 and 12 months, of migraine patients continuing treatment with single-pulse transcranial magnetic stimulation

	Baseline	Month 3		Month 12	
	Median (IQR)	**Median (IQR)**	**Wilcoxon Signed ranks test**	**Median (IQR)**	**Wilcoxon Signed ranks test**
Headache days	18.0 (13.0 - 30.0)	11.0 (5.0 - 24.0)	*P* < 0.001	12.0 (5.0 - 20.0)	*P* < 0.001
Migraine days	13.0 (9.0 - 23.0)	8.0 (3.0 - 13.25)	*P* < 0.001	6.0 (3.0 - 11.0)	*P* < 0.001
Headache free days	4.0 (0.0 - 13.0)	6.0 (0.0 - 17.5)	*P* = 0.041	12.0 (0.0 - 12.0)	*P* = 0.003
Abortive treatment intake days	9.0 (4.0 - 14.0)	4.0 (0.0 - 9.0)	*P* = 0.005	4.0 (2.0 - 9.0)	*P* = 0.014
Headache Impact Test-6	66.0 (65.0 - 68.0)	60.0 (56.0 - 64.0)	*P* < 0.001	62.0 (56.0 - 65.0)	*P* < 0.001

Headache-related disability

Compared to baseline, the reduction of mean HIT-6 score was 4 points at month 12, from 66 (IQR: 64.0-69.0), to 62 (IQR: 56.25-65.0); ($P < 0.001$, $r = -0.51$, Fig. 61E). The percentage of patients with severe headache-related disability was reduced from 93% at baseline to 63.3% at month 3 and 62.9% at month 12. Furthermore, 20.0% at month 3 and 24.1% of patients at month 12 reported some or little/ no headache impact compared to 4.2% at baseline (Table 13).

Table 13: Changes in headache impact test-6 headache disability categories after daily single-pulse transcranial magnetic stimulation treatment for all patients

	Baseline (*N* = 143) *N* (%)	**Month 3 (*N* = 96) *N* (%)**	**Month 12 (*N* = 54) *N* (%)**
Severe impact (60-78)	133 (93.0%)	62 (64.6%)	34 (63.0%)
Substantial impact (56-59)	4 (2.8%)	15 (15.6%)	7 (13.0%)
Some impact (50-55)	5 (3.5%)	15 (15.6%)	10 (18.5%)
Little or no impact (<48)	1 (0.7%)	4 (4.2%)	3 (5.5%)

Subgroup analysis

Further analysis of subgroups within the 153 patients looked at potential differences in the MHD at month 3 between; (1) migraine with aura and migraine without aura patients, (2) pre- BoNT/A patients and patients which had previously failed BoNT/A treatment, (3) patients classified as MOH and non-MOH (4) patients diagnosed with vestibular symptoms.

There were no significant differences in MHD at month 3 or month 12 in any subgroups analysed (**Aura *vs* non-aura**; Month 3: $P = 0.524$, $r = -0.09$, Month 12: $P = 0.919$, $r = -0.02$. **Post-BoNT/A *vs* pre-BoNT/A**; Month 3: $P = 0.139$, $r = -0.21$, Month 12: $P = 0.665$, $r = -0.08$. **MOH *vs* non-MOH**; Month 3: $P = 0.637$, $r = -0.07$, Month 12: $P = 0.082$, $r = -0.36$. **With vs without vestibular symptoms**; Month 3: $P = 0.796$, $r = -0.06$, Month 12: $P = 0.624$, $r = -0.16$) (Table 14).

Safety and tolerability

During the 3-month sTMS trial, (15.0 %, *N* = 23/153) (ITT: 11%, 23/214) of patients reported at least one side effect. Beyond 3-month sTMS trial 4 further patients (17.6 %, *N* = 27/153) (ITT: 13%, 27/214) reported side effects. Overall, the most frequent adverse events were: discomfort at the side of the sTMS delivery (34.3 %, *N* = 12/35), worsening

of the headache (28.6 %, *N* = 10/35), and nausea (11.4 %, *N* = 4/35). Adverse events were transient, lasting for seconds to minutes following sTMS stimulation and described as mild or moderate in the vast majority of patients. One patient stopped sTMS treatment due to scalp sensitivity.

Table 14: Subgroup analysis of mean headache days at baseline and after three months treatment with single-pulse transcranial magnetic stimulation

	Baseline *median (IQR)*	**Month 3** *median (IQR)*	**Wilcoxon Signed ranks test**	**Month 12** *median (IQR)*	**Wilcoxon Signed ranks test**
With aura (*N* = 65)	16.0 (11.0 - 26.0)	12.0 (5.0 - 22.25)	*P* = 0.085	11.0 (5.3 - 24.8)	*P* = 0.134
Without aura (*N* = 75)	18.0 (12.0 - 29.0)	14.0 (3.0 - 20.0)	*P* = 0.017	13.5 (5.0 - 17.0	*P* < 0.001
Mann-Whitney	*P* = 0.308	*P* = 0.524		*P* = 0.919	
Pre-BoNT/A (*N* = 62)	16.5 (10.0 - 26.0)	12.0 (5.0 - 18.0),	*P* = 0.002	9.0 (4.0 - 18.0),	*P* = 0.090
BoNT/A (*N* = 80)	18.0 (13.25 - 28.0)	14.0 (8.0 - 24.5),	*P* = 0.010	14.0 (5.0 - 19.5),	*P* = 0.010
Mann-Whitney	*P* = 0.500	*P* = 0.139		*P* = 0.665	
MOH (*N* = 65)	18.0 (13.0 - 23.0)	13.0 (8.5 - 18.50	*P* = 0.003	9.0 (5.0 - 16.0)	*P* = 0.004
Non-MOH (*N* = 78)	17.5 (10.75 - 30.0)	13.0 (5.0 - 29.5),	*P* = 0.029	14.5 (4.3 - 24.5)	*P* = 0.038
Mann-Whitney	*P* = 0.503	*P* = 0.637		*P* = 0.082	
With vestibular symptoms (*N* = 26)	16.5 (10.0 – 30.0)	12.0 (4.0 – 22.5)	*P* = 117	9.0 (3.0 – 20.5)	*P* = 0.083
Without vestibular symptoms (*N* = 117)	18.0 (12.5 – 25.0)	13.0 (6.25 – 24.0)	*P* = 0.005	11.0 (5.0 – 18.0)	*P* = 0.001
Mann-Whitney	*P* = 0.465	*P* = 0.796		*P* = 0.624	

BoNT/A, Botulinum Toxin Type A; **MOH,** Medication overuse headache; **IQR,** interquartile range

Chapter 5:
sTMS clinical audit

Discussion

This is the first large independent prospective analysis evaluating the effectiveness and tolerability of sTMS in difficult-to-treat CM or high frequency migraine patients with and without aura patients with and without MOH. Furthermore, this is the first non-invasive neuromodulation long-term analysis conducted in headache disorders. Our findings suggest that sTMS may be a safe and effective treatment in this population with sustained benefit overtime. Furthermore, it demonstrates that sTMS is safe and well tolerated, with only a very small proportion of patients discontinuing the treatment because of adverse events. We found no significant outcome differences in patients with MOH and BoNT/A non-responders, supporting a place in clinical practice for a broad spectrum of migraine patients.

The management of chronic and high frequency migraine remains challenging for reasons linked to effectiveness, tolerability, treatment delivery modalities and costs. Regardless of the treatment's mechanisms of action, the reported responder rate of treatments traditionally used for migraine prophylaxis ranges between 40-60%, even when the more migraine specific calcitonin gene related peptide (CGRP) pathway is tackled (Dodick et al., 2018a), leaving a significant proportion of patients with insufficient symptoms management. Additionally, tolerability issues linked to poor adherence of oral pharmacological treatments means that frequent medications switching is often necessity before a tolerated treatment is found (Hepp et al., 2014, Rahimtoola et al., 2003). Injectable treatments namely Onabotulinum toxin A (BoNTA) and the CGRP monoclonal antibodies (mAB's) have partly resolved the tolerability issues, displaying a better profile than oral medicines in clinical trials (Xu et al., 2019). However, the former treatment with regular three-monthly administration regime, may put long-term unsustainable pressure on headache clinics due capacity issues, leaving some patients with lack of treatment continuity, whereas the latter novel treatment lack of long-term data in CM (Lambru et al., 2020, Andreou et al., 2020, Deng et al., 2020). sTMS seems to overcome these traditional treatment obstacles by displaying an excellent tolerability profile and by reducing the need of outpatient clinical appointments, which adds on the long-term favourable cost-effective profile (Dodick et al., 2010).

The vast majority of patients treated in this audit would meet the recently updated EHF criteria for resistant CM since they failed at least three drug classes with evidence in migraine prevention and a significant minority of patients would meet the definition of refractory CM (Sacco et al., 2020). About half of these patients, were considered responders to sTMS at month 3, mainly due to significant reduction in mean MHD, MMD, which likely led to headache-related disability improvement. Furthermore, we noticed that almost a third of patients experienced a meaningful reduction in headache-related disability without clear-cut improvement in the other measured headache characteristics ("headache disability responders"). Of this group, 78% continued to feel much improved at one-year follow-up. Although not rigorously measured, it is possible that reduction in headache duration may have contributed to this outcome. Our findings were less impressive than a previous 3-month study were a higher responder rate and a more profound reduction in mean MMD was observed (Starling et al., 2018). It is possible that this difference reflected the more complex and difficult-to-treat group of patients treated in this audit. Similarly, to the previous report, a significant impact on QoL has been reported in our sTMS responders, suggesting that this treatment has a role in improving what ultimately matters for migraine patients, which is headache-related quality of life.

sTMS resulted in sustained improvements in one third of the whole patient group (60% of the 3-month responders) at one-year follow-up. Eight of these patients even completed two years follow-up, supporting its treatment role in chronic migraine patients. In these patients, a sustained reduction in mean MHD and MMD and HIT-6 was observed at 1 year follow-up. Furthermore, sTMS led to a progressive reduction of MOH patients from 52% at baseline to 8% at month 12, suggesting a potential effect also in the group of CM with MOH patients.

Poor sleep quality is known to be an exacerbating factor for migraine attacks (Fernández-de-Las-Peñas et al., 2018) other migraine preventative treatments including amitriptyline have been shown to improve sleep quality for migraine patients (Duman et al., 2015). TMS may also have a beneficial effect for patient sleep (Nardone et al., 2020). rTMS has been shown to improve sleep quality in subjects with insomnia (Jiang et al., 2013), (Feng et al., 2019), restless leg syndrome (Altunrende et al., 2014), (Lin et al., 2015), sleep

bruxism (Zhou et al., 2016). sTMS has been shown to have positive clinical outcomes as an add-on therapy for patients with severe depression (Conca et al., 2000) while some studies have found no significant difference in sleep disturbance they also suggest that the TMS might influence the characteristics of sleep allowing for "a sense of profound sleep" (Fujita and Koga, 2005). This could explain why patients who did not see a 30% reduction in headache days none the less continued with the treatment and reported an improvement in quality of life.

sTMS showed a very favourable safety and tolerability profile in our patients, with very few patients reporting adverse events and one patient only discontinuing the treatment because of them. The excellent tolerability profile of sTMS has been reported in all previous studies (Dodick et al., 2010), making it an appealing treatment option in migraine patients who struggle to tolerate medicines.

The main limitation of this audit is the open label design. However, it is unlikely that the long-term symptoms improvement could be explained by placebo alone. The strengths of this report include the refractoriness of the group of patients treated, which reflect the type of complex and difficult-to-treat patients seen in tertiary headache clinics and the long follow-up, which give essential clinical information for treatments that are supposed to be given long term. Ultimately, the prospective and the real-world nature of the analysis, which includes patients not subject to strict inclusion and exclusion criteria.

In conclusion, sTMS was effective in the prevention of migraine symptoms in a meaningful proportion of treatment resistant CM patients with and without MOH. sTMS' s beneficial effect consisted in reduction in traditional headache efficacy measures, namely monthly headache and migraine frequency, but also in improvement of headache related QoL. The improvement was sustained overtime in the majority of our patients and not influenced by the level of patients' refractoriness. Along with the excellent tolerability profile, this data suggests that sTMS has a place in the preventive treatment of chronic/high frequency migraine.

Chapter 6: General discussion

Experimental Findings

The studies reported in this thesis aimed to investigate the mechanism of action of single-pulse transcranial magnetic stimulation (sTMS) as an acute and preventative treatment for migraine. Animal models were used to directly investigate the acute and chronic effects of sTMS on key brain structures associated with migraine, including; direct modulation of cortical activity and indirect modulation of thalamic and hypothalamic activity. Additionally, to investigate sTMS in a real-world scenario, an open label clinical audit was carried out of patients using sTMS for migraine. This chapter will discuss the mechanisms of action of sTMS. Then it will compare the results of our experiments with previous research, before considering the implications for clinical use of sTMS. This chapter will then consider the study limitations and suggest future study extensions.

Mechanisms of action of sTMS

This section will outline the mechanisms of action of our sTMS studies. Experimental evidence in this thesis has demonstrated that direct, acute, subthreshold application of sTMS to the occipital cortex can inhibit neuronal activity in the cortex and increase the threshold for initiation of CSD (Fig. 62A). This inhibition can be blocked by GABA antagonists (Fig. 62D). Reduced cortical excitation could provide a possible mechanism for how sTMS is able to increase the electrical threshold required to produce CSD and why the peak neuronal activity during a CSD wave is significantly smaller after sTMS (Fig. 62B & C). In addition to directly modulating neuronal activity in the cortex, sTMS also induces efferent cortical modulation of neuronal activity in deeper brain nuclei related to migraine, particularly causing inhibition of spontaneous and induced activity of the thalamus (Fig. 62E & F).

Finally, daily long-term application of sTMS accumulates, having a lasting effect beyond the acute effects previously shown. This daily long-term sTMS application inhibits the excitability of the directly affected cortex and indirectly affected cortico-thalamic networks. Such changes are not observed on second order neurons (Fig. 62H). The corticothalamic pathway from the visual cortex is robust, providing circular activation and modulation; in comparison, the visual cortex has much less of a corticospinal pathway. This suggests that sTMS' top-down modulation is limited to the cortex and cortically connected structures and does not have a direct stimulating impact on peripheral cephalic and dural trigeminal fibres.

Chapter 6:
General Discussion

I believe that the key finding is that the main mechanism of action in sTMS is via modulation of inhibitory cortical GABAergic interneurons. These cells have been suggested to have a lower activation threshold well below the motor and phosphene thresholds (Ilić et al., 2002), meaning they can be activated by a lower strength magnetic pulse without stimulating cortical pyramidal cells as has been suggested previously.

This helps to explain all the other findings presented in this thesis. Activation of GABAergic interneurons in laminae II and III would inhibit excitatory neurons in those laminae with intercortical projections in lamina I. This inhibition would explain the altered thresholds and properties of CSD seen. Although laminae V and IV are not thought to contain inhibitory interneurons, they receive inputs from superficial laminae that do. Increased inhibition in superficial laminae (II, III and IV) would have knock on effects, such as a reduced input to pyramidal neurons in lamina V. Decreased input to lamina V, would explain the reduced spontaneous activity as was directly recorded using electrophysiology. Promoting inhibition in the cortex would change the corticothalamic pathway to switch from high frequency to low frequency firing. As well as reducing direct feedback to the thalamus, low frequency cortical firing would also promote thalamic inhibition, as was observed, through inhibition from the thalamic reticular formation.

Chapter 6:
General Discussion

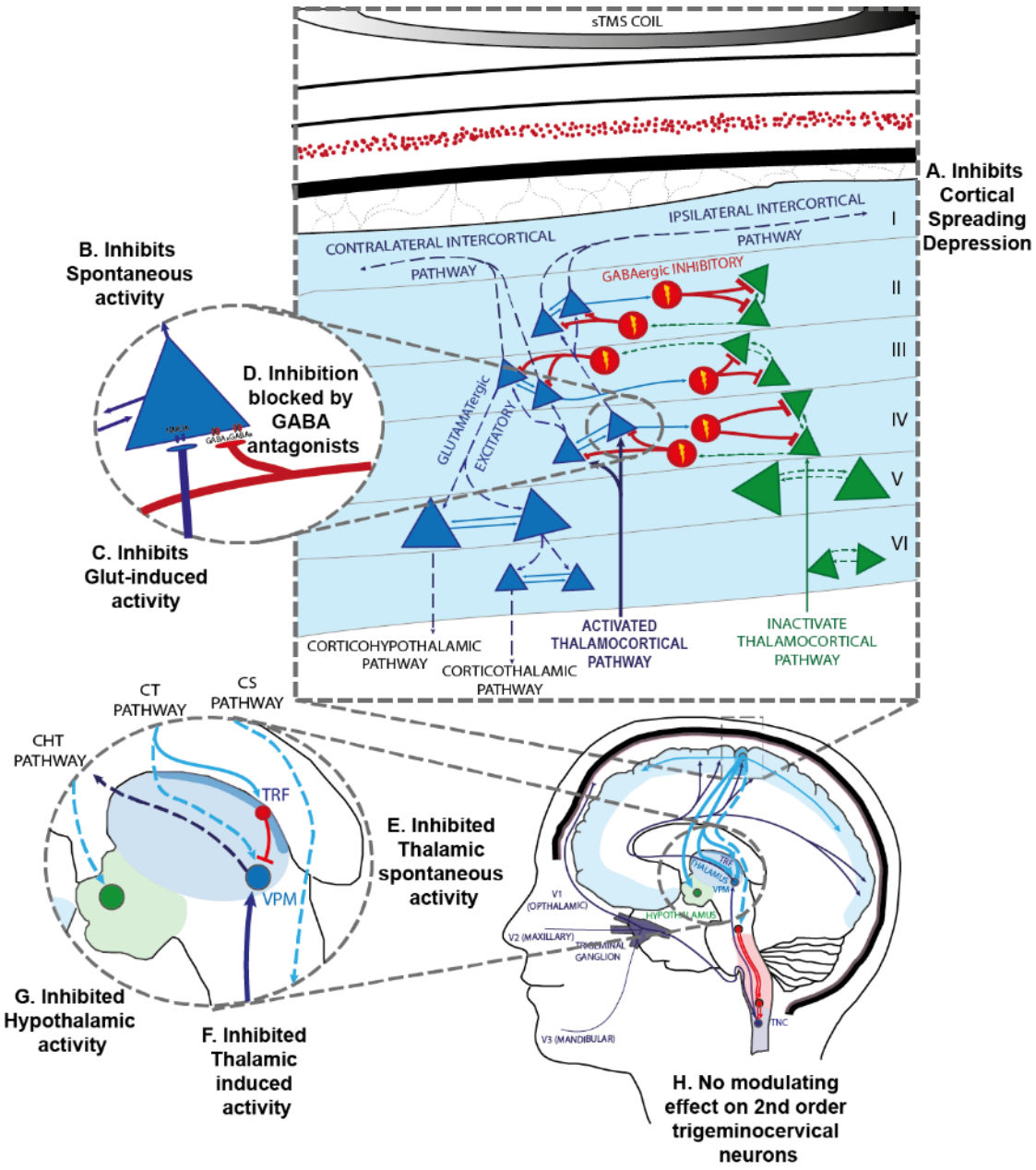

Figure 62: Simplified Summary of findings for the effects of 1.1 T sTMS and proposed site of action (*A) Electrical threshold for CSD increased (B-D) Enlarged view of simplified single excitatory cell body (B) Spontaneous activity of the cortex reduced in a dose-dependent fashion (C) cortical glutamate-induce activity reduced (D) Inhibition of glutamate-induce activity and increased CSD thresholds blocked in the presence of GABA antagonists (E-G) Enlarged view of diencephalon (E+F) Descending corticothalamic (CT) projections from lamina VI inhibit spontaneous and induced ventroposterial medial (VPM) thalamic activity, possibly through recruitment of inhibitory thalamic reticular formation (TRF) neurons (G) Hypothalamic activity inhibited by CSD and 1.1 T sTMS from descending projections of the corticohypothalamic (CHT) pathway (H) No sensitisation of second order neurones in the trigeminal nucleus caudalis (TNC) from the Corticospinal (CS) pathway seen following long-term application of sTMS. Proposed mechanism is that subthreshold sTMS pulses stimulate low threshold inhibitory cortical GABAergic interneurons (in red) in laminae II – IV, inhibiting excitatory cortical neurons reducing activity internally within the cortical column and externally along intercortical, corticothalamic and corticohypothalamic pathways.*

Chapter 6:
General Discussion

Comparison with previous research

Having explained the mechanisms of action of our sTMS studies; in this section, we will consider how our studies compare with previous research. These findings fit with previous research investigating the mechanisms of sTMS. Andreou *et al.* (2016) found that sTMS significantly inhibited both mechanical and chemically induced cortical spreading depression, when administered immediately post-induction in rats. However, they found that administration of sTMS pre-induction was not able to inhibit CSD induction. The post-induction inhibition of CSD concurs with our finding of increased threshold for electrical CSD induction. However, we saw this inhibition when sTMS was applied prior to CSD initiation.

Andreou *et al*. found less of an effect of sTMS on CSD induction in cats compared with rats, which may suggest that sTMS is less effective in mammals with more complex gyrencephalic brains, which has implications for human treatment. They proposed this species difference was due to a larger cortical area recruited with the 25 mm coil in rats, as opposed to cats, as well as additional features such as the thicker cat skull and thicker cortex. In this thesis we showed that a smaller diameter sTMS coil (11 mm) produced consistent and significant inhibition of CSD in rats. This may suggest that it is the depth of penetration and not the area of affect that influences the extent of cortical inhibition. However, as well as cortical structural differences between the species there are also functional differences. AMPA receptor antagonists and GABA receptor agonists have been shown to be less effective at inhibiting CSD induction in cats than rats (Holland et al., 2010), which may suggest the species difference is unrelated to sTMS activity.

Murphy *et al.* (2015) suggested that sTMS activates inhibitory $GABA_B$ receptors in the upper cortical layers, which in turn inhibit activity of dendritic pyramidal neurons in layer V of the somatosensory cortex. By blocking $GABA_B$ receptors they were able to prevent inhibitory effects of sTMS on the somatosensory cortex (Murphy et al., 2016). We also found that $GABA_B$ (and $GABA_A$) antagonists prevented inhibition of glutamate induced spontaneous activity of lamina V, and increased threshold of CSD induction. GABAergic inhibition has also been suggested as a mechanism for sub-threshold paired pulse TMS in several studies (Funke and Benali, 2010, Ilić et al., 2002) and shown in computer modelling of sTMS action on the cortex (Esser et al., 2005).

sTMS also inhibited spontaneous and C-fibre activity of third order neurons in the VPM thalamic nucleus. This thalamic inhibition was previously found to be blocked by pre-treating with the broad μ-opioid receptor antagonist, naloxone, suggesting that sTMS could interact with the endogenous opioidergic system to produce its anti-cephalalgic effect (Andreou et al., 2016). The naloxone was believed to be acting in the cortex, rather than the thalamus or spinal cord, which would also fit with the sTMS' action on cortical GABAergic interneuron inhibition.

Finally, Andreou *et al.* also showed that sTMS has no modulating effect on the firing of second order trigeminocervical neurons (Andreou et al., 2016), which we also showed during the long-term application of sTMS, supporting our belief that sTMS' effect is limited to the cortex and brain structures robustly connected to the cortex.

Implications for Clinical Utilisation

Having compared our studies to other research; this section will consider the implications for clinical use of sTMS to treat migraine patients. Following a successful clinical trial showing efficacy in migraine (Lipton et al., 2010), and several post-marketing studies (Bhola et al., 2015, Starling et al., 2018, Irwin et al., 2018), sTMS has been approved for the treatment of migraine by the National Institute for Health and Care Excellence (NICE, 2014b). The Headache centre at Guy's and St Thomas' NHS Trust was the first NHS tertiary headache clinic to offer sTMS, staring in 2017.

A clinical audit of the first 153 patients using sTMS as a preventive treatment was carried out and reported in chapter 6. During the clinical audit, patients demonstrated an improvement of the primary endpoint (number of headache days per month) as well as the secondary endpoints (number of migraine days per month, number of days abortives were used and headache impact test score). Fifty seven percent of patients opted to continue using sTMS beyond the 3-month trial period. Of them, 45 patients, who were not considered responders, opted to continue using sTMS. No patients reported serious side effects

Subgroups within the treated patient population (including patients with medication overuse headache, BoNT/A failure, aura symptoms and vestibular symptoms) did not identify any groups that had significantly better or worse outcomes using sTMS. The lack of difference between migraine with and without aura patients is somewhat surprising, given the inhibition of the cortex seen during the animal experiments. Given migraine with aura patients have more excitable cortex, the expectation was that inhibition caused by the sTMS would produce a greater therapeutic effect. Unfortunately, the headache diaries filled in by patients did not monitor aura symptoms, so it was not possible to observe any effects of sTMS on the presence or quality of auras. This may be an interesting symptom to consider monitoring in the future, to explore the real-world validity of the experimental findings.

Current guidelines suggest that sTMS be used as a tertiary prophylactic medication once BoNT/A has been trialled and failed, meaning at least 4 previous prophylactic treatments have also been tried and failed. Treatment with sTMS has been shown to be more cost effective than BoNT/A injections (Brüggenjürgen et al., 2016). BoNT/A has proved to be a safe treatment for migraine prophylaxis (Diener et al., 2010), however, it is a neurotoxin and although unlikely, side effects from BoNT/A can be potentially severe, including partial facial paralysis. sTMS has the advantage of having no severe side effects and relatively few minor side effects (Dodick et al., 2010). Given the efficacy, cost-effectiveness and safety, sTMS could be adopted earlier in the migraine pathway, before BoNT/A trial, as a secondary or tertiary treatment option.

Several studies have suggested that there may be age differences in the extent of modulation by sTMS. Paired pulse TMS to the motor cortex (70% motor threshold) was shown to cause increased inhibition in middle aged participants (56.1 years ± 4.9) compared with younger patients (28.5 years ± 5.2) (Kossev et al., 2002). Peinermann *et al.* (2001) also showed differences in paired-pulse TMS inhibition on the motor cortex (75% motor threshold); however, they found a reduction in the magnitude of intracortical inhibition in the older population (mean; 51 years) than the younger group (mean ;28 years) (Peinemann et al., 2001). Age differences could have important ramifications for the treatment of migraine patients as the condition is present from teenage years to old age. This finding may also suggest that sTMS treatment would be more viable in middle

aged patients. Both studies were carried out in the motor cortex of healthy volunteers, it is unclear if these findings can be generalised to other cortices or applied to migraine patients. In the current clinical audit, patient age was not a subgroup variable that was analysed, this was mainly due to the available n numbers, which would be too low due to the multiple age groups. The audit is ongoing and there are plans for a follow up audit once patient numbers at the 1-year and 2-year mark increase, this may be an interesting factor to analyse when investigating the effectiveness of sTMS across the patient population.

Prophylactic treatments for migraine (including; topiramate, divalproex sodium, nortriptyline, and propranolol) did not appear to alter the effect of sTMS in migraine with aura patients (Almaraz et al., 2010). This study was limited to only 164 migraine with aura patients and grouped all prophylactic treatments rather than investigating each individually. Other studies investigating drug interactions with TMS have focused on TMS as an exploratory tool, especially stimulating the motor cortex to find a motor threshold as a measure of excitability. A comprehensive literature review was carried out by Ziemann *et al.* in 2004 and revisited in 2015. It found that voltage-gated sodium channel antagonists (such as lamotrigine, phenytoin, and carbamazepine) increase the motor threshold, NMDA receptor antagonists (ketamine) decrease the motor threshold and drugs acting on GABA receptors (including; benzodiazepines, vigabatrin and valproic acid) do not influence the motor threshold (Ziemann et al., 2015, Ziemann, 2004). How relevant these findings are to interactions with sTMS applied to the occipital cortex in migraine patients requires further investigation. Currently there is no evidence for drug-device interaction that may influence the efficacy of sTMS as an acute or preventative treatment for migraine. To confirm this, further research is required to fully understand the sites of action of sTMS and how it would interact with cortically available pharmaceuticals.

This thesis suggests that from the stimulating locus in the cortex, sTMS drives a reduction in cortical, hypothalamic and thalamic activity. In corroboration with previous research (Andreou et al., 2016), it also suggests sTMS application has minimal effect on peripheral primary afferent fibres of the trigeminal nerve. Combining sTMS with a treatment whose action is on peripheral pathways, such as BoNT/A, could prevent both the central and

peripheral mechanisms that drive migraine attacks, producing a greater effect than either treatment in isolation. Clinically, sTMS is a cost-effective treatment that deserves proper funding within the NHS.

Experimental Limitations

The experimental chapters of this thesis used animal models to investigate the effect of sTMS on brain structures associated with migraine. There are several limitations to be addressed with this choice of model.

In this thesis, animal models of Sprague Dawley rats and GCaMP mice were used to model the effects of sTMS. Both rats and mice are lissencephalic, with smoother and less complex cerebral cortexes than larger mammals like cats, dogs and of course humans, that are gyrencephalic with folded cerebral cortexes. CSDs are easier to induce in lissencephalic animals and spread across the entire flat hemisphere of the cortex, rather than the spread being limited by the sulci in gyrencephalic brains (Kenny et al., 2019). Additionally, the propagation velocity of the CSD wave is more consistent in lissencephalic brains, whereas gyrencephalic brains show deceleration as the wave crosses a sulcus (Bowyer et al., 1999). Therefore, how much of the finding can be translated to humans requires further investigation.

Migraine is a complex, chronic condition, with underlying genetic and plastic components that predispose the patients to developing migraine attacks. These studies were carried out on naïve animals without these elements. An example to illustrate this is the model of CSD used. The mechanical and electrical stimulations used to generate CSD during these experiments are invasive and damaging to the cortical tissue. While they are the best available model, producing a reliable, consistent CSD at will, they are not the spontaneously generated migraine aura, driven by innate cortical hyperexcitability, present in humans.

Migraine predominantly affects females, with approximately 17% of the female and 6% of male population affected. However, in all experiments using rats in this thesis, males were utilised. Hormonal fluctuation is known to play an important role in migraine.

Chapter 6:
General Discussion

Migraine attacks peak on the first day of the cycle (MacGregor, 2015, Fox and Davis, 1998b), with an inverse relationship to the level of oestrogen (MacGregor, 2004). However, how much of a role hormonal fluctuation would play in the central mechanisms investigated here remains unclear. Using female animal models would be preferential, in order to be more relevant to the condition being studied, however, that would require additional monitoring and analysis of the stage of the oestrous cycle. This would be costly in terms of the time and number of animals required to complete the experiments. Males were used as hormonal levels stay broadly consistent over time.

Despite the limitations associated with these animal models they are none the less still considered to be the best available choice (Andreou and Oshinsky, 2015). The procedures are too invasive for human subjects, which would also preclude the collection of necessary tissue samples. There are currently no models that replicate all aspects of the migraine condition, from the spontaneous chronic nature, to the premonitory phase or the sensory disturbances that arise during the attack. But individual aspects of migraine have been modelled well and are considered a reliable tool to be used for scientific investigation (Andreou and Oshinsky, 2015).

In the electrophysiological experiments outlined in this thesis, a dilute solution of 60 mg/kg pentobarbital sodium was used to anaesthetise the animals. This anaesthetic choice was used as it has been previously shown that pentobarbital affects CSD initiation and propagation equivalent to the lowest concentrations of volatile anaesthetics (Kitahara et al., 2001, Kudo et al., 2013, Kudo et al., 2008). In addition pentobarbital was used to be consistent with previous research (Andreou et al., 2016). Pentobarbital sodium is a short acting barbiturate used as an anaesthetic and sedative, acting primarily as an agonist of $GABA_A$ receptors to enhance inhibitory GABA action (Suckow et al., 2012). As we have shown that sTMS appears to have interactions with GABAergic mechanisms, the use of pentobarbital could limit the effect of sTMS on cortical mechanisms seen. This could be improved with the use of non-GABAergic anaesthetics, for example ketamine, however these have been shown to have a greater effect on cortical functioning. Ideally, the acute effects of sTMS on the cortex should be evaluated on non-anaesthetised awake animals, this could be achieved with an implanted microelectrode array recording from awake

animals (Baker et al., 1994). However, as this would require an additional level of ethics approval, such experiments are not feasible in our department.

Study Extensions

Three areas for further study have already been identified in the previous two sections of this chapter. First, that a clinical trial for the preventive treatment of migraine with sTMS, that includes aura symptoms in headache diaries is performed. Such a trial would investigate whether the effect of sTMS varies on migraines with and without aura. Second, that the effect of age on sTMS be investigated, through an ongoing clinical trial with larger age subgroups. Third, although so far there are no such indications, investigation into the potential effect of drug-device interaction, to determine whether drug treatment could increase the efficacy of sTMS would be valuable for the field.

In this section, continuing from the experiments in this thesis, there are several other interesting research directions that could be explored further, to expand our understanding of the mechanisms of action of sTMS as a treatment for migraine.

The main suggestion put forward by this thesis is that sTMS achieves modulation through activation of cortical GABAergic interneurons. However, activity of these cells have not been directly observed, only inferred through their interaction with excitatory pyramidal cells and the blocking of GABAergic receptors on those cells. So, the first study extension proposed is direct observation of cortical GABAergic interneurons during sTMS treatment. Direct recording of inhibitory interneurons could be achieved via the use of electrophysiology and patch-clamping; however, this would be *ex vivo*, recording cortical cell in isolation of the entire system. Another technique that could be utilised is intensity-based GABA sensing fluorescence reporter (iGABASnFR) mouse model (Marvin et al., 2019) and *in vivo* calcium imaging to directly record cortical GABA activity in real time.

This thesis has demonstrated that with long-term sTMS application, there is modulation to the cortex and thalamus beyond the acute effect previously seen, suggesting a cumulative effect. However, further exploration would be required to elucidate how it achieves this effect and how long these effects remain for. Therefore, the second study extension proposed is an investigation into the long-term, cumulative effects of sTMS

treatment. Determining if the long-term effects of sTMS are a continuation of the acute effects could be achieved by investigating antagonism of the cortical GABAergic system. Would blocking GABAergic interneurons, throughout the long-term application of sTMS, negate the cumulative inhibition of the cortex and thalamus? Unfortunately, this presents its own problems, the GABA antagonists (as were used in chapter 3) are proconvulsant (Treiman, 2001) and thus cannot be applied globally or over a prolonged period to the cortex.

One of the major questions when investigating treatments for migraine in general, is why some patients prove resistant to treatment? In the clinical audit, 56% continued using the sTMS treatment beyond the 3-month trial, with no significant differences in the subgroups analysed, consistent with previous research. Therefore, the other 44% of patients were not receptive to treatment. This could be due to an, as yet unknown interpatient variation in stimulation thresholds for inhibitory interneurons that may determine the success of sTMS treatment. The issue of non-responders applies not just to treatment with sTMS but all currently available acute and preventative treatments. 30% of patients do not respond to a single oral triptan (Ferrari et al., 2002) and ~30% of patients were not considered responders after 3 rounds of BoNT/A treatment (Silberstein et al., 2015). Understanding why this is the case is vitally important for targeting existing treatments to those that would receive the greatest benefit, extending existing treatments to a greater percentage of patients and developing new treatments that are effective in more patients. There are many aspects of the pathophysiology of migraine that still remain unknown, which limits our complete understanding of how we may effectively prevent and treat migraine attacks, therefore further research is required. So, the third study extension proposed is an investigation into the pathophysiology of migraine.

Chapter 6:
General Discussion

Conclusion

In conclusion, the pre-clinical observations in this thesis further contribute to the advancement of our understanding of how sTMS modulates neuronal activity of key brain areas associated with migraine, in order to prevent and treat migraine attacks. This may ultimately contribute to using the existing treatment more effectively and making improvements to the technique to better treat migraine.

Results from this thesis illustrate that acute application sTMS modulated activity of the cortex it was directly applied to. sTMS causes inhibition of spontaneous and glutamate induced cortical neuronal activity. sTMS also alters activity of cortical spreading depression, increasing the threshold of activation and altering properties of the wave once initiated. The observations suggest the modulation of excitatory cortical neurons via the recruitment of inhibitory GABAergic systems. sTMS' effect on glutamate activity and CSD threshold is negated with the use of GABA antagonists. These finding provide a better understanding for how sTMS acts on the cortex to achieve an acute reduction in headache pain. Inhibiting activity within the cortex also has secondary effect on cortically connected brain structures causing reduced activity in the thalamus and hypothalamus. Reducing thalamic activity may disrupt incoming trigeminal spinal signals that drive a migraine attack. These acute effects on the cortex and thalamus accumulate with long term application, with implications for how sTMS can act as a preventative treatment. Long term cortical application of sTMS also does not appear to cause sensitisation of the TCC. This would suggest that prolonged use would not develop into a peripheral sensitisation equivalent to medication overuse headache.

Finally, sTMS has been shown to be an effective treatment in a real-world scenario. A clinical audit of patients using sTMS found an overall improvement in patient outcomes with a significant number of patients continuing the treatment for at least 12 months.

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