Characterisation of Low-Intensity Repetitive Transcranial Magnetic Stimulation Induced Neuroplasticity in Rodent and Experimental Models

Alexander Dinesha Tang (B.Sc. Hons.)

Experimental and Regenerative Neurosciences, School of Animal Biology

This thesis is presented for the degree of Doctor of Philosophy at The University of Western Australia

August 2016
Abstract

Recent developments in non-invasive brain stimulation, especially repetitive transcranial magnetic stimulation (rTMS), have opened up the possibility of modifying neuroplasticity by means of extrinsic transient magnetic fields. The physiological mechanisms underlying rTMS-induced plasticity in humans and whether the regions exposed to low intensity stimulation contribute to the overall plasticity induced with rTMS is unknown. Rodent and experimental models provide a useful adjunct to human rTMS studies due to the more direct and often invasive techniques used to assess changes in plasticity. This thesis will characterise the effect of low-intensity rTMS (LI-rTMS) on neuroplasticity using neurotrauma and healthy rodent and ex vivo models (brain slices) to determine whether LI-rTMS is capable of neuromodulation. In addition, rodent-specific rTMS coils will be developed and characterised in order to deliver focal stimulation for the LI-rTMS studies conducted in healthy intact nervous tissue. 12 milli-Tesla (mT) LI-rTMS, previously shown to induce axonal growth after a partial CNS injury, was delivered to mice with a complete optic nerve crush to investigate potential neuroprotective and axonal regenerative effects of LI-rTMS, evaluated with histological and molecular paradigms. Two novel rodent-specific coil designs, both 8mm in diameter were designed to deliver focal LI-rTMS, approximately 100mT in intensity. The physical properties (e.g. magnetic field, temperature etc.) were characterised alongside electric field modelling and biological testing with potential changes in motor evoked potentials in anaesthetised rats. Changes in behaviour of healthy adult animals were assessed by pairing daily skilled motor learning with LI-rTMS either as priming or consolidating stimulus for ten consecutive days. Western blot and ELISAs were used to investigate changes in key synaptic proteins and BDNF 24 hours after the last rTMS and training session. To investigate potential mechanisms underlying LI-rTMS-induced changes in neural excitability, whole-cell patch clamp recordings were made from the soma of layer 5 pyramidal neurons in motor and somatosensory mouse brain slices. Passive and active membrane properties were measured before low-intensity repetitive magnetic stimulation (LI-rMS) and at 0, 10 and 20 minutes post-stimulation. Our results show that 12 mT LI-rTMS does not induce neuroprotection or axonal regeneration after a
severe injury to the optic nerve. The novel rodent-specific coils deliver 120mT stimulation, induce a maximum electric field of 12.7V/m and increase MEP amplitudes in anaesthetised rats with 10Hz LI-rTMS. Using these coils, LI-rTMS as a priming stimulus transiently increased skill accuracy but did not alter the rate at which mice learned the motor task. At the single cell level, LI-rTMS did not alter the passive membrane properties of excitatory neurons but increased neural excitability by hyperpolarising the action potential threshold and increasing the evoked spike firing frequency, with the onset of changes occurring immediately after stimulation. The results from this thesis demonstrate the ability of LI-rTMS to induce neuroplasticity in healthy but not severely injured nervous systems. We suggest that LI-rTMS in its own right has significant neuromodulatory effects which may contribute to the overall plasticity induced by rTMS.
Student contribution declaration

DECLARATION FOR THESES CONTAINING PUBLISHED WORK AND/OR WORK PREPARED FOR PUBLICATION

The examination of the thesis is an examination of the work of the student. The work must have been substantially conducted by the student during enrolment in the degree.

Where the thesis includes work to which others have contributed, the thesis must include a statement that makes the student’s contribution clear to the examiners. This may be in the form of a description of the precise contribution of the student to the work presented done by the student.

In addition, in the case of co-authored publications included in the thesis, each author must give their signed permission for the work to be included. If signatures from all the authors cannot be obtained, the statement detailing the student’s contribution to the work must be signed by the coordinating supervisor.

This thesis contains published work and/or work prepared for publication, some of which has been co-authored. The bibliographical details of the work and where it appears in the thesis are outlined below. The student must attach to this declaration a statement for each publication that clarifies the contribution of the student to the work. This may be in the form of a description of the precise contributions of the
student to the published work and/or a statement of percent contribution by the student. This statement must be signed by all authors. If signatures from all the authors cannot be obtained, the statement detailing the student’s contribution to the published work must be signed by the coordinating supervisor.

Sections of the introduction were published in The Neuroscientist:

Chapter 2 was published in Plos One: Tang, A.D., Makowiecki, K., Bartlett, C. and Rodger, J., 2015. Low intensity repetitive transcranial magnetic stimulation does not induce cell survival or regeneration in a mouse optic nerve crush model. PloS One, 10(5), p.e0126949. ADT contribution : 85%


Chapter 4 has been prepared for submission :Tang, A.D., Bennett, W., Collins, J., Hadrill, C., Wills, K., Puri, R., Garry, M.I., Hinder, M.R., Summers, J.J., Rodger, J. and Canty, A.J., 2016. 120mT Intermittent theta burst rTMS primes but does not consolidate skilled reaching in mice. Manuscript prepared ADT contribution:70%
Chapter 5 has been accepted in *Neuroscience*: Tang, A.D., Hong, I., Boddington, L.J., Garrett, A.R., Etherington, S., Reynolds, J.N.J and Rodger, J., 2016. Subthreshold repetitive magnetic stimulation lowers action potential threshold and increases spike firing in layer 5 pyramidal neurons in vitro. *Neuroscience*. Accepted. ADT contribution: 95%

Sections of the introduction and discussion were submitted to *Frontiers in Neural Circuits*: Tang, A.D. and Vallence AM., 2016. Human inter-individual variability to repetitive transcranial magnetic stimulation: how animal and experimental models can help. *Frontiers in Neural Circuits*. Under revision. ADT contribution: 60%

.................................................................
List of figures

Chapter 1

Figure 1.1. Basic principles of TMS.

Figure 1.2. Biphasic vs monophasic waveforms.

Figure 1.3. Simple and patterned rTMS frequencies

Figure 1.4. Modelling of round coils and figure of 8/butterfly coils show different electric field distributions.

Figure 1.5. Commercial coils used to deliver rTMS to rodents.

Figure 1.6. Induced current distribution with high intensity rTMS.

Figure 1.7. Modelling of the membrane potential of a pyramidal neuron undergoing single pulse TMS

Figure 1.8. “Cooperative” mechanism of rMS-induced LTP at the excitatory synapse in the hippocampus.

Chapter 2

Figure 2.1. Diagrammatic representation of the study design.

Figure 2.2. LI-rTMS does not affect RGC survival or axonal regeneration following optic nerve crush.

Figure 2.3. LI-rTMS does not modulate BDNF.

Chapter 3

Figure 3.1. Schematic diagrams of coils used in rodents, the novel rodent-specific coils and the induced waveforms.

Figure 3.2. Characterisation of coil properties.

Figure 3.3. Finite element modelling of the iron-core coil.
Figure 3.4. Finite element modelling of the Magventure BC-65HO butterfly coil.

Figure 3.5. Characterisation of MEP’s before and after 10Hz rTMS to the anaesthetised rat motor cortex with the iron-core coil.

Chapter 4

Figure 4.1. Priming and consolidation LI-rTMS on skilled motor behaviour.

Figure 4.2. Western blot analysis of cortical tissue from animals receiving priming LI-rTMS and motor training.

Figure 4.3 Mean BDNF expression assessed 24 hours after the last priming rTMS and motor training session

Chapter 5

Figure 5.1. In vitro LI-rMS delivery setup and representative traces of evoked spiking.

Figure 5.2. LI-rMS hyperpolarises AP threshold and increases spike firing.

Figure 5.3. LI-rMS does not alter the passive membrane properties, AP spike shape
List of tables

Chapter 2

Table 2.1 RGC survival and BDNF concentrations following stimulation.

Chapter 3

Table 3.1 Dielectric properties used in modelling.

Appendix

Table A1. Summary table of raw values for chapter 5.

Table A2. Summary of p values from repeated measures ANOVA’s of raw values for chapter 5.

Abbreviations

ACSF- Artificial Cerebrospinal Fluid
AHP- After Hyperpolarisation
AMPA receptor- α-amino-3-hydroxyl-5-methyl-4-isoxazolepropionic receptor
ANOVA- Analysis of Variance
AP- Action Potential
ARC- Activity-Regulated-Cytoskeleton-associated protein
BDNF- Brain Derived Neurotrophic Factor
BK- Big Potassium Channels
CNS- Central Nervous System
cTBS- Continuous Theta Burst Stimulation
dB- Decibel
EDTA- Ethlenediaminetetraacetic Acid

ELFMs- Extremely Low Frequency Magnetic Fields

ELISA- Enzyme linked Immunosorbent Assay

EMG- Electromyography

FEM- Finite Element Modelling

GABA<sub>A</sub> receptor- Gamma-Aminobutryic Acid A Receptor

GAD65- Glutamate Decarboxylase 65

GAP43- Growth Associated Protein 43

Glur1- Glutamate Receptor 1

Glur2- Glutamate Receptor 2

HI-rTMS- High Intensity Repetitive Transcranial Magnetic Stimulation

Hz- Hertz

ISI- Inter-stimulus Interval

iTBS- Intermittent Theta Burst Stimulation

K<sub>A</sub> channel- A type Potassium channel

LI-rMS- Low-Intensity Repetitive Magnetic Stimulation

LI-rTMS- Low-Intensity Repetitive Transcranial Magnetic Stimulation

LTD- Long Term Depression

LTP- Long Term Potentiation

MEP- Motor Evoked Potentials

mRNA- Messenger Ribonucleic Acid

MSO- Machine Stimulator Output

NMDA receptor- N-methyl-D-Aspartate Receptor
PBS- Phosphate Buffered Solution

PIPES- Piperazine-N,N-bis(2-ethanesulfonic acid)

PNS- Peripheral Nervous System

PSD95- Post-synaptic Density 95

RGCs- Retinal Ganglion Cells

QPS- Quadripulse Stimulation

RMP- Resting Membrane Potential

rMS- Repetitive Magnetic Stimulation

RMT- Resting Motor Threshold

rTMS- Repetitive Transcranial Magnetic Stimulation

SEM- Standard Error of the Mean

SK channels- Small Potassium channels

SPL- Sound Pressure Level

tACS- Transcorneal Alternating Current Stimulation

TBS- Theta Burst Stimulation

tES- Transcorneal Electrical Stimulation

TMS- Transcranial Magnetic Stimulation

Trk-B receptor- Tyrosine Kinase B receptor

VGSC- Voltage Gated Sodium Channels
Acknowledgements

I would like to thank my supervisors and collaborators for their support and guidance during my PhD. In particular, I would like to thank A/Prof Jenny Rodger for her amazing mentorship and encouragement over the last 4 years. I am extremely grateful for her tireless commitment and the many training and travel opportunities she has provided.

I am deeply grateful to my family and friends for their encouragement and support through a challenging but rewarding thesis. I would also like to thank my fellow lab mates at EaRN for the light hearted and enjoyable work environment they created.

This work was funded by the National Health and Medical Research Council of Australia, the Australian Research Council and the Neurotrauma Research Program of WA. Moreover, I would like to thank the Australian Government, the University of Western Australia and the Bruce and Betty Green Foundation for providing doctoral scholarship.
# Table of Contents

Abstract ......................................................................................................................... i

Student contribution declaration .................................................................................. iii

List of figures .................................................................................................................. vi

List of tables ..................................................................................................................... viii

Abbreviations .................................................................................................................... viii

Acknowledgements .......................................................................................................... x

Chapter 1: Introduction .................................................................................................. 1

1.1 Neural Plasticity ......................................................................................................... 1

1.1.2 Hebbian plasticity .................................................................................................. 1

1.1.3 Metaplasticity ....................................................................................................... 2

1.1.4 Brain derived neurotrophic factor (BDNF) in plasticity ........................................ 3

1.1.5 Inducing neural plasticity non-invasively ............................................................... 4

1.2 Electromagnetism ...................................................................................................... 4

1.2.1 History of CNS stimulation with electricity and magnets ...................................... 5

1.3 Transcranial Magnetic Stimulation .......................................................................... 5

1.4 rTMS .......................................................................................................................... 6

1.4.1.1 Stimulus waveform ......................................................................................... 7

1.4.2 rTMS frequencies ............................................................................................... 8

1.4.3 TMS coils ............................................................................................................ 10

1.4.4 Intensity .............................................................................................................. 14

1.5 rTMS induced plasticity .......................................................................................... 15

1.5.1 Changes in corticospinal excitability ................................................................... 16

1.5.2 Change in motor learning and behaviour ............................................................. 16

1.6 rTMS mechanisms .................................................................................................... 18
1.6.1 Where on the neuron does TMS act? ................................................................. 18
1.6.2 Synaptic mechanisms .................................................................................... 20
1.6.3 Differential effects on inhibitory and excitatory networks ......................... 23
1.6.4 Non-synaptic mechanisms .......................................................................... 25
1.6.5 rTMS at the systems level ............................................................................ 26
1.6.6 rTMS as a treatment for neurotrauma ....................................................... 28
1.6.7 High intensity vs low intensity rTMS-induced plasticity mechanisms ...... 30

Aims ...................................................................................................................... 30

Aim 1: rTMS as a potential therapy for neuroprotection and axonal regeneration .31
Aim 2: Development of higher intensity rodent-specific rTMS coils ................. 33
Aim 3: Characterisation of rTMS on skilled motor learning .............................. 34
Aim 4: Investigation of rTMS on neural excitability at the single cell level ....... 35

Chapter 2: Low intensity repetitive transcranial magnetic stimulation does not induce

cell survival or regeneration in a mouse optic nerve crush model....................... 36

Introduction ........................................................................................................ 36

Methods ............................................................................................................. 38

Animals ............................................................................................................... 38
Optic nerve crush ............................................................................................... 38
LI-rTMS ............................................................................................................. 39
Tissue preparation ............................................................................................. 40
Immunohistochemistry ...................................................................................... 40
Stereological analysis of retinal wholemounds .............................................. 41
Enzyme-Linked Immunosorbent Assay (ELISA) and Protein Assay ............. 41
Statistical Analysis ............................................................................................. 42

Results ............................................................................................................. 43
LI-rTMS does not increase RGC survival......................................................... 43
LI-rTMS does not induce RGC axon regeneration ......................................... 43

xii
LI-rTMS does not increase BDNF in the retina or optic nerve ........................................44
Discussion.................................................................................................................46
Does intensity matter?.................................................................................................46
LI-rTMS effects may be brain region specific.........................................................47
Non-invasive brain stimulation techniques for treating neurotrauma .................49
Conclusion.................................................................................................................50

Chapter 3: Construction and Evaluation of Rodent-Specific rTMS Coils .............51
Introduction ...............................................................................................................51
Methods ......................................................................................................................52
Coil and stimulation parameters .............................................................................52
Magnetic field decay and measurements ...............................................................53
Field strength during 1 and 10 Hz stimulation ......................................................53
Temperature measurements ..................................................................................53
Sound measurements ..............................................................................................53
Finite element modelling .......................................................................................54
Anaesthesia and Electromyography ....................................................................56
Single Pulse TMS and rTMS ..................................................................................57
Data analysis ............................................................................................................58
Results .......................................................................................................................59
Magnetic field strength – peak values and decay .................................................59
Changes in coil temperature ..................................................................................59
Sound emission from coils ....................................................................................60
Magnetic field stability ............................................................................................60
Finite element modelling .......................................................................................61
10Hz rTMS and cortical excitability .......................................................................63
Discussion .................................................................................................................64
Chapter 4: Low-intensity repetitive magnetic stimulation modulates skilled motor learning in mice.

Introduction ..........................................................................................................................69
Methods .................................................................................................................................71
Animals ........................................................................................................................................71
Custom rodent rTMS coil ........................................................................................................71
Repetitive Transcranial Magnetic Stimulation ........................................................................72
Skilled Motor Training: Single-Pellet Reaching Task ...............................................................72
Molecular Biology Tissue Preparation ..................................................................................73
Western Blotting .....................................................................................................................73
BDNF ELISA ............................................................................................................................74
Data Analysis and Statistics ......................................................................................................74
Results ........................................................................................................................................75
Skilled reaching motor skill .....................................................................................................75
Speed of reaching did not increase with either time or treatment ............................................76
Molecular Analysis of Priming LI-rTMS Cortical Tissue .........................................................77
Discussion ...................................................................................................................................79

Chapter 5: Low-intensity repetitive magnetic stimulation lowers action potential threshold and increases spike firing in layer 5 pyramidal neurons in vitro. ................84

Introduction .............................................................................................................................84
Methods .......................................................................................................................................85
Ethics approval ..........................................................................................................................85
Slice preparation .........................................................................................................................85
Electrophysiology .....................................................................................................................86
Repetitive magnetic stimulation (rMSS) ....................................................................................87
Data analysis ..............................................................................................................................87
Statistical analysis ....................................................................................................................88
rMS induces a hyperpolarised AP threshold and increases spike firing frequency...89
rMS does not alter passive membrane properties, spike shape properties or fast after-hyperpolarisation.................................................................................90
Discussion........................................................................................................91
Chapter 6: General Discussion ........................................................................96
What’s new? .......................................................................................................96
Mechanisms of low-intensity rTMS-induced plasticity.................................96
Low-intensity rTMS-induced plasticity may be cell/region specific ..............98
Delivering focal rTMS to rodents .................................................................99
Is low-intensity stimulation clinically irrelevant? ........................................100
Limitations of rodent and experimental models compared to human TMS ...102
Conclusion........................................................................................................103
References.........................................................................................................105
Appendix ..........................................................................................................129
Chapter 1: Introduction

1.1 Neural Plasticity

Plasticity of the central nervous system (CNS) (i.e. neural plasticity) is traditionally described as the ability of the CNS to rearrange its functional and anatomical connections in response to environmental cues, thereby achieving new or altered functions (Dunlop, 2008). A fundamental principle of neuroscience, plasticity plays a significant and dynamic role, not only in the healthy CNS (e.g. learning and memory) but also in neurological disease and disorders.

Changes in plasticity are governed by multiple mechanisms and encompass changes at the molecular, structural and behavioural level in addition to the most well-known mechanism, changes in synapse strength (synaptic plasticity). The ability to non-invasively induce and modulate plasticity has generated great interest and underlies the therapeutic aims of non-invasive brain stimulation. The most characterised and applied non-invasive brain stimulation technique to modulate plasticity in humans is repetitive transcranial magnetic stimulation (rTMS), which is the technique investigated in this thesis. The introduction of thesis will provide an overview of (i) neural plasticity principles, (ii) a history of electromagnetism, (iii) rTMS and its parameters (iv) rTMS-induced plasticity, (v) rTMS-induced plasticity mechanisms and (v) the experimental aims.

1.1.2 Hebbian plasticity

In the search to explain the mechanisms underlying memory and learning, Donald Hebb postulated “When an axon of cell A is near enough to excite cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A’s efficiency as one of the cells firing B, is increased.” (Hebb, 1949). This was later simplified and summarised as “cells that fire together wire together” and suggests that synapses are able to store a memory of previous activity. Experimental evidence to support Hebb’s postulate was provided by Bliss and Lomo who showed that high frequency electrical stimulation of the rabbit hippocampus led to an increase in excitatory post-synaptic potential amplitudes (Bliss and Lømo, 1973), which they termed “long-term potentiation” (LTP).
Although Hebb did not postulate the inverse process of LTP, i.e. that synapses could be weakened via long term depression (LTD), such a process is needed to optimise information in a neural network (Sjöström et al., 2008) and was postulated by Gunther Stent: “When the presynaptic axon of cell A repeatedly or persistently fails to excite postsynaptic cell B while cell B is firing under the influence of other presynaptic axons, metabolic change takes place in one or both cells such that A’s efficiency, as one of the cells firing B, is decreased.” Or put simply- “cells that do not fire together do not wire together”. Evidence supporting the concept of LTD also comes from the hippocampus, but this time through the use of low frequency stimulation (Bramham and Srebro, 1987). Taken together with the studies investigating LTP, synaptic plasticity can superficially be thought of as changes in synaptic strength and can be induced experimentally with high frequency stimulation (LTP) and low frequency stimulation (LTD).

1.1.3 Metaplasticity

Since the discovery of synaptic plasticity over 50 years ago (Bliss and Lømo, 1973), there is now a wealth of evidence that implicates LTP and LTD as the mechanisms underlying learning and memory (Martin et al., 2000, Neves et al., 2008) and essential players of neuroplasticity. While synaptic plasticity is generally considered as a beneficial phenomenon, the mechanisms controlling synaptic plasticity and limiting its saturation are equally as important in order to prevent extreme levels of synaptic plasticity (e.g. excessive weakening or strengthening of synapses) and even CNS damage (e.g. excitotoxicity) (Abraham, 2008). This “plasticity of plasticity” is known as metaplasticity (Abraham and Bear, 1996), and is “a change in the physiological or biochemical state of neurons or synapses that alters their ability to generate synaptic plasticity” (Abraham, 2008). Metaplasticity incorporates multiple mechanisms, many of which overlap with synaptic plasticity. One such mechanism is the history of synaptic activation, whereby the levels of pre and post synaptic activity can alter the threshold for LTP and LTD induction. For example, previous periods of high synaptic activity, or external stimulation with high frequency stimulation can increase the threshold for subsequent LTP or decrease the threshold for LTD and vice versa (Frey et al., 1995, Huang et al., 1992, Mockett et al., 2002). Therefore, whilst synaptic plasticity
is a core concept of neuroplasticity, techniques that modulate neuroplasticity are likely to interact with both synaptic and metaplasticity.

1.1.4 Brain derived neurotrophic factor (BDNF) in plasticity

In addition to causing changes in synaptic strength and neuronal electrical properties, plasticity can occur at the molecular level. One molecule that plays a crucial role in plasticity is brain derived neurotrophic factor (BDNF). BDNF is a member of the “classic” neurotrophin family and provides trophic support for both the peripheral and central nervous system and is crucial during the development and ongoing plasticity of the nervous system (Park and Poo, 2013). Clinically, BDNF has therapeutic potential for both neurodegenerative diseases (Nagahara et al., 2009, Nagahara and Tuszynski, 2011) and for regeneration in neurotrauma (Tobias et al., 2003, Blesch and Tuszynski, 2007).

BDNF is synthesised from a precursor isoform (Pro-BDNF) (Matsumoto et al., 2008), which is stored in secretory vesicles ready to be released by the neuron in an activity-dependent manner (Mowla et al., 2001, Poo, 2001). After release, Pro-BDNF may be converted to mature BDNF by proteolytic cleavage of the Pro-domain by plasmin or metalloproteinases (Nagahara and Tuszynski, 2011) and is a ligand for the tyrosine kinase receptor type B (TrkB) (TRK family) (Soppet et al., 1991) and for the p75 receptor (tumour necrosis factor family) (Carter, 1996). Mature BDNF has high affinity for the TrkB receptor, whilst Pro BDNF has high affinity for p75 receptor. Activation of the TrkB receptor promotes neuronal survival by initiating signalling pathways to suppress pro-apoptotic proteins such as the MEK-ERK pathway and activation of anti-apoptotic proteins (Kaplan and Miller, 2000). Activation of the MEK-ERK pathway is known to also influence neurite outgrowth, cellular differentiation and neuronal survival during development and continues to provide neuronal support in the adult CNS (Nagahara and Tuszynski, 2011). The p75 receptor can act as a co-receptor for the TrkB receptor and potentiate the effects of TrkB activation, but predominantly activates signalling pathways that induces apoptosis (Kaplan and Miller, 2000, Koshimizu et al., 2010). Activation of the p75 receptor is also necessary during development for axon pruning (Cao et al., 2007, Singh et al., 2008). BDNF trophic support has been well established to be essential for proper CNS development and continues its support into the mature CNS, influencing dendritic
branching (Horch and Katz, 2002) and synaptic plasticity (Bramham and Messaoudi, 2005).

1.1.5 Inducing neural plasticity non-invasively

Since Hebb’s postulate, there has been a great deal discovered on synaptic and non-synaptic plasticity, most of which was discovered and characterised in animal and experimental models. Although studies continue to investigate the basic mechanisms underlying plasticity in the healthy nervous system, there is great interest in developing novel therapies that can modulate plasticity mechanisms in order to induce or modify neural plasticity to treat neurological diseases and disorders. Non-invasive brain stimulation is an example of such a therapy and has become extremely popular as it can induce LTP and LTD “like” plasticity in clinical and non-clinical populations (Hoogendam et al., 2010, Pelletier and Cicchetti, 2015). However, despite its widespread use, the mechanisms underlying non-invasive brain stimulation-induced plasticity remains unclear. Moreover, investigating how non-invasive brain stimulation modulates plasticity in humans is difficult as directly assessing plasticity is often invasive. As such, animal and experimental preparations make for ideal models in which to investigate the physiological mechanisms of non-invasive brain stimulation-induced plasticity as advanced and invasive techniques can be used.

1.2 Electromagnetism

Although Michael Faraday is considered to be the father of electromagnetism due to his pivotal work on the application and effects of electricity and magnetism in both chemistry and physics, it was Danish physicist Hans Oested who first discovered the relationship between electricity and magnetism. In 1820, Oested observed that a compass needle would deviate when a current flowed through a nearby wire (Kipnis, 2005). A decade later, Faraday showed that a temporary voltage could be induced in a nearby secondary wire if a current was passed through the primary wire. The induction of this voltage/eddy currents in the second conductor is a result of the transient magnetic field induced by a current passing through the primary wire and is summarised by Faraday’s law of electromagnetic induction. More specifically, the transient magnetic field (B) induces an electric field (E) in the second conductor which produces the current (I). The mathematical relationship between the transient
magnetic field and the induced electric field was formalised by physicist James Maxwell (Maxwell, 1892), who showed that the induced electric field is proportional to rate of change in magnetic field intensity over time (i.e. \( E \propto dB/dT \)). This phenomenon is most commonly exploited for industrial and mechanical applications such as power generators and transformers, but given the high electrical conductivity of nervous tissue, Faraday’s induction can also be used to non-invasively stimulate the brain.

1.2.1 History of CNS stimulation with electricity and magnets
The use of electricity to stimulate the brain and alter neurological function or disease dates back to the Roman empire in 43 AD, where physicians would use the electrical currents produced by torpedo fish as a treatment for headaches and gout (Pascual-Leone and Wagner, 2007). In the 1700’s, Galvani demonstrated that electrical stimulation could be used to stimulate nervous tissue, by observing muscle responses following electrical stimulation of isolated frog nerves (Galvani, 1791). However the use of electromagnets to stimulate the brain developed much later (late 1800s and early 1900s) with the earliest studies showing that magnetic pulses over the brain induced phosphenes (Pascual-Leone and Wagner, 2007). This later developed into the use of magnetic stimulation to non-invasively stimulate peripheral nerves in both animals and humans (Bickford and Femming, 1965), where the authors suggested that stimulation was a result of eddy currents induced in the vicinity of motor nerves (Pascual-Leone, 1999). However the routine use of magnetic stimulation was hindered by the poor quality of stimulator equipment at the time which was prone to overheating and structural instability. This was later overcome by Anthony Barker and colleagues, who are often referred to as the founders of modern transcranial magnetic stimulation (TMS).

1.3 Transcranial Magnetic Stimulation
Transcranial magnetic stimulation (TMS) was first described in 1985 (Barker et al., 1986) and has become one of the most common forms of non-invasive brain stimulation. The biophysical mechanisms of TMS are underpinned by Faraday’s principle of electromagnetic induction, whereby a stimulation coil is placed over the cranium to deliver a time-varying electromagnetic field resulting in an induced electrical current in the underlying cortex (figure 1.1). The induced current is used to stimulate the underlying nervous tissue, and if sufficient in intensity, can induce action
potentials. Barker and colleagues used this principle to non-invasively stimulate the human motor cortex resulting in movement of the leg or arm (Barker et al., 1985). This TMS-induced motor activity can be measured with surface electrodes to measure the motor-evoked potential (MEP) of the target muscle. Single pulse TMS and MEPS are useful in diagnostics as the latency of the MEP provides a measurement of CNS conduction velocity (Barker et al., 1986) and the amplitude can be used as a measure of cortical excitability (Hoogendam et al., 2010). Whilst the majority of TMS studies involve the motor cortex due to the ease of MEP measurements, single pulse TMS can also be used to temporarily disrupt neuronal activity to induce “virtual lesions”, providing a useful tool to determine the function of certain brain regions (Pascual-Leone, 1999).

Figure 1.1. Basic principles of TMS. A brief current is pulsed through a magnetic coil overlying the brain, inducing a magnetic field over the brain. Through electromagnetic induction, the magnetic field induces an electric field and current underneath the coil resulting in neural stimulation. By Lenz’s law, the direction of the current in the induced conductor (i.e. the brain) flow in the opposite direction of the current that flows in the coil.

1.4 rTMS

Single pulse TMS provides a useful diagnostic tool for both clinical and non-clinical populations. However, TMS can also be used to modulate neural plasticity by applying multiple or repetitive TMS pulses, better known as repetitive TMS (rTMS). This approach has therapeutic potential in neurological disease and disorders (e.g. stroke, Parkinson’s disease, depression etc.)

At the heart of all rTMS stimulator equipment is a power source that delivers programmable current pulses to a stimulator. Within this simple design exists multiple factors which can be altered to produce different biological changes and include (i)
stimulus waveform/pulse shape, (ii) rTMS frequency, (iii) rTMS coils and (iv) stimulation intensity.

1.4.1.1 Stimulus waveform

The two most common waveforms used in TMS and rTMS are classified as monophasic (waves consisting of a single polarity such as half a sine wave) and biphasic pulses (consisting of two polarities such as a sine wave) (figure 1.2). The choice of monophasic or biphasic pulses originally developed due to the design limitations of monophasic stimulators commonly used in basic and single pulse TMS stimulators. In monophasic stimulators, magnetic pulses are generated from a complete discharging of a charged capacitor whereby ~50% of discharged energy is used to generate the magnetic field and the other 50% dissipated as heat. Monophasic stimulators are designed to deliver either only positive or only negative voltage through the coil. The shortcoming of this design is that the capacitor needs to be completely recharged before the next pulse/stimulus can be delivered. Therefore delivering multiple stimuli of short inter-stimulus intervals (ISI) (e.g. theta-burst stimulation ISI = 20ms) is not possible when the capacitor must be recharged from zero voltage (Wassermann et al., 2008). Using biphasic waveforms overcomes this limitation by using capacitors that can deliver positive and negative charge, allowing the energy not used in generating the magnetic field of the first pulse to be stored and available for the next pulse, reducing the capacitor re-charge time (Wassermann et al., 2008).

The stimulus waveform or shape of the generated magnetic field is an important factor not only from a practical point of view but from a functional view as biphasic and monophasic pulses shape have been shown to induce different outcomes in both single pulse TMS and rTMS (Taylor and Loo, 2007, Sommer et al., 2006, Arai et al., 2005, Kammer et al., 2001). In single pulse TMS, monophasic pulses are less efficient and need to be delivered at a greater intensity than biphasic pulses to reach MEP threshold (i.e. to induce a MEP) (Sommer et al., 2006, Kammer et al., 2001). Differences in MEP threshold are explained by the differences in the induced electric field/current profile between monophasic and biphasic pulses. Unlike monophasic pulses, biphasic pulses induce a second phase on the induced electric field profile, resulting in a greater change to the membrane potential (Davey and Epstein, 2000) and hence stimulated neurons reach threshold with lower stimulation intensities compared
to monophasic pulses. However in rTMS, monophasic pulses have been shown to induce greater plasticity than biphasic pulses (Arai et al., 2005, Taylor and Loo, 2007, Arai et al., 2007). These differences in the physiological responses to rTMS may be due to the differences in neuronal populations activated by monophasic and biphasic pulses, where monophasic activates more uniform neuronal populations and biphasic activates multiple populations which may cancel each other out (Arai et al., 2007, Arai et al., 2005) (discussed below).

1.4.2 rTMS frequencies

The pattern in which rTMS pulses are given, or rTMS frequency, is measured in hertz (Hz) and is often described as “simple” or “complex”. Similar to synaptic plasticity, the frequency at which stimulation is delivered is a significant factor on determining the type of plasticity induced (e.g. facilitation or depression) (Hoogendam et al., 2010).

1.4.2.1 Simple frequencies

Simple frequencies are defined by the constant ISI that occurs between pulses (e.g. 1Hz = 1 pulse per second= ISI of 1s) (figure 1.3). Stimulation with simple frequencies can be further divided into “high” and “low” frequency stimulation, with “low” frequency stimulation defined as ≤1Hz and high frequency stimulation defined as > 5Hz (Hoogendam et al., 2010). Although still frequently used, the popularity of simple
frequencies is rivalled by the use of complex and patterned stimulation frequencies due to the development of more advanced TMS stimulators (Suppa et al., 2016).

1.4.2.2 Complex and patterned frequencies

Complex and patterned frequencies contain pulses with variable ISIs (e.g. theta burst stimulation = 3 pulses with an ISI of 20ms repeated every 200ms (Hoogendam et al., 2010) (figure 1.3). Interestingly, the most popular complex protocols used are the theta burst stimulation (TBS) protocols, intermittent TBS (iTBS) and continuous TBS (cTBS) (Huang et al., 2005). TBS is based on an endogenous oscillatory neuronal firing pattern detected in the hippocampus with bursts repeated every 4-7 Hz (theta range in electroencephalography) during exploratory behaviour of rats (Diamond et al., 1988). TBS was subsequently used to induce LTP experimentally with electrical stimulation, where 100Hz pulse trains repeated at 5Hz (i.e. theta rhythm) induced robust synaptic plasticity (Capocchi et al., 1992, Larson et al., 1986). Hypothesising that this protocol would also be effective in humans, Huang and colleagues adapted the experimental TBS protocol to fit the capabilities of rTMS stimulators of the time which were capable of delivering 3 pulses of 50Hz, repeated every 5Hz (Huang et al., 2007). This magnetic pulse form was further divided into iTBS, where TBS is delivered in 2s intervals followed by an 8 second gap or cTBS where TBS is delivered without spacing intervals. Since its introduction in 2005, TBS protocols have become extremely popular as they are thought to be powerful and induce more reproducible plasticity than other rTMS protocols, a claim which is still under debate (Suppa et al., 2016).

Building on the rationale of TBS, Hamada and colleagues started to explore other experimental protocols in which the number of pulses in a complex train of stimulation is known to modulate synaptic plasticity in the hippocampus (Nakao et al., 2004). Their complex frequency “quadrupulse stimulation” (QPS) protocol, is an extension of the work by Thickbroom et al. which showed that trains of 2 pulses with an ISI of 1.5ms, repeated every 5 seconds increased cortical excitability (Thickbroom et al., 2006). Hamada and colleagues modified the protocol by delivering trains of 4 pulses with an ISI of 1.5 or 5ms with the trains repeated every 5Hz (Hamada et al., 2008).
Figure 1.3. **Simple and patterned rTMS frequencies.** Examples of simple rTMS frequencies (top), where the inter-stimulus intervals are constant (i.e. 1Hz = 1 pulse/second = inter-stimulus interval of 1s). Patterned rTMS frequencies (bottom) gave more complex inter-stimulus intervals such as theta burst stimulation where three pulses at 50Hz are repeated at 5Hz (i.e. every 200ms), or quadripulse where trains of 4 pulses are delivered with an inter-stimulus interval of 1.5 or 5ms and repeated at 5Hz. Figure adapted from (Hoogendam et al., 2010).

### 1.4.3 TMS coils

In humans, TMS can be delivered with a variety of commercial coils with the figure of 8 and round coil designs being the most commonly used. Round coils are the simplest design with the resulting magnetic field is greatest directly under the centre of the coil, whilst the induced current is greatest at the edge of the coil underneath the wire (Wassermann et al., 2008). Despite their small size, circular coils are relatively unfocal. Focality is increased when using figure of 8 coils (also known as butterfly coils) which consist of two circular coils placed side by side such that the current flow at the junction point is in the same direction. This configuration results in the summation of the induced electromagnetic field at the junction point, creating a “hot-spot” (Wassermann et al., 2008) (figure 1.4).
Figure 1.4. Modelling of round coils and figure of 8/butterfly coils show different electric field distributions. Round coils consist of 1 coil with multiple turns of wire (A). Figure of 8 also known as butterfly coils, consist of two round coils placed closely together (B). The round coils induce a maximum electric field/current underneath the windings (C) whereas the maximum electric field in a figure of eight coil occurs at the edge where the two round coils meet and is used to increase stimulation focality (D). Figure adapted from (Deng et al., 2013).

Ideally, TMS coils would be capable of delivering both focal and high intensity stimulation but in practice, this combination is difficult to achieve, because increasing stimulation intensity is at the expense of focality and vice versa (Deng et al., 2013). Coils can be optimised to produce greater magnetic field strength, and therefore greater induced current, by winding the wire around a core made of a ferromagnetic material such as iron (Epstein and Davey, 2002). In addition, an iron core increases field penetration (Deng et al., 2013) allowing for the stimulation of deeper structures. Optimising coils for focality typically involves reducing coil size, to limit the area of stimulated cortex. However, scaling down coil geometry requires larger currents or an increased number of wire turns to achieve high intensity magnetic fields and results in greater thermal and mechanical stress (Cohen and Cuffin, 1991). The problem is exacerbated in rTMS, where multiple pulses of high intensity magnetic fields/currents are delivered in quick succession.

In small animal and experimental models, the choice of TMS coils is extremely limited, with most studies using commercial human coils to deliver high intensity TMS at the expense of focality in the small rodent brain. Nonetheless, some degree of focality can be achieved in rats as evidenced by the ability to elicit unilateral MEPs (stimulation of
only one cortical hemisphere) within a certain range of intensity (Rotenberg et al., 2010) (figure 1.5A).

Recent animal and in vitro studies using commercial human TMS stimulators have significantly increased our understanding of the mechanisms that underpin TMS-induced plasticity, however the lack of focal stimulation in experimental models, equivalent to that induced by human coils, prevents direct translation to clinical studies (Weissman et al., 1992, Wassermann and Zimmermann, 2012). One approach to improve focality is the placement of a shield plate under human stimulator coils, limiting the electromagnetic field imposed on the cranium (Gasca et al., 2010). Whilst computer modelling suggests increased focality, further investigation into the induced thermal and mechanical stresses is needed. The most obvious solution for improved focality (spatial resolution) is to down scale coil size and designing coils more appropriate for animals. Recent attempts at producing stronger animal specific coils to stimulate deep brain structures have led to novel coil designs, such as the halo coils (March et al., 2013, March et al., 2014). However, the strong electromagnetic fields induced by these coils are again at the cost of focality (Deng et al., 2013). This is more evident in the recent work by Parthoens and colleagues who recently characterised and commercialised a small rTMS coil for rats (Parthoens et al., 2016). The small round coil (40mm outer diameter) is capable of suprathreshold stimulation and induces electric fields in rats that are similar to human TMS applications (figure 1.5) but has proved to be unsuitable for focal stimulation. While the authors were able to overcome thermal instability by coupling the coil to a cooling device (circulating fluid around the coil), electric field modelling suggests that the coil delivers bilateral stimulation. Furthermore, when the coils were used to induce MEPs, coil placements over the cortex and spinal cord resulted in MEPs similar in latency, suggesting that the coil is still relatively unfocal and results in spinal cord activation even when placed towards the cortex. Therefore, whilst this rat-specific coil can deliver suprathreshold stimulation, the simultaneous stimulation of both brain and spinal cord makes it difficult to determine where any rTMS-induced plasticity might have occurred.

An alternative is to deliver highly focal TMS with very small implantable coils, for example sub-millimetre coils (500µm diameter transducers) can deliver suprathreshold stimulation in vitro (Bonmassar et al., 2012) and in vivo (Park et al., 2013). Although
not completely analogous to traditional TMS (the coil is implanted beneath the skull), such micro-coils allow delivery of chronic focal magnetic stimulation in non-restrained and possibly awake animals.

If focality is prioritised over magnetic field intensity it is possible to build rodent sized coils that retain focality. Recent studies from our laboratory have used mouse-specific coils (8mm circular coils) to deliver rTMS. These coils deliver an rTMS intensity of a magnitude three times less than human stimulators, avoiding mechanical and thermal instability. Using these coils, we have had success in inducing plasticity \textit{in vitro} and in the mouse visual system using low-intensity rTMS and I further explore the applications of these low intensity coils in an injury model in chapter 2. However, the mechanisms underlying these plastic changes clearly differ to those evoked using high-intensity rTMS in clinical application (Grehl et al., 2015). Therefore, there exists a gap in our ability to investigate rTMS-induced plasticity at different intensities in animal and experimental models as the current options are to either stimulate at high intensity with low focality or at low intensity with high focality. This limitation will be addressed by designing and testing novel coils in chapter 3 of this thesis.

\textbf{Figure 1.5. Commercial coils used to deliver rTMS to rodents.} Human figure of 8 coils can be offset to achieve suprathreshold stimulation restricted to one hemisphere to deliver rTMS and single pulse TMS (A). Commercial rat coil consisting of a curved 40mm diameter round coil, shaped to fit around the rat head (B) capable of inducing MEPs and stable high frequency rTMS but overlies the entire rat brain (C) and both right and left hemispheres (D). Figure consists of images from (Rotenberg et al., 2010) and (Parthoens et al., 2016).
1.4.4 Intensity

Unlike waveforms and frequencies which have been extensively investigated as determinants of rTMS effects, the contributions of intensity remain poorly understood. Stimulation intensity is a key factor in rTMS as it not only affects the spatial distribution of the electromagnetic field but also the amplitude of the current induced in the brain (e.g. suprathreshold or subthreshold currents) which are likely to induce different plasticity mechanisms. As described above, the rate at which a transient magnetic field is generated and collapses (dB/dT) is proportional to the magnitude of the electric field induced in the brain and is in turn proportional to the magnitude of the current induced in the brain (Wassermann and Zimmermann, 2012). Therefore, if the rise and fall times of the magnetic field remain constant but the absolute magnitude of the magnetic field increases, a larger electric field and resulting current is induced in the brain. This intensity can be scaled to deliver subthreshold or suprathreshold stimulation, where suprathreshold stimulation results in depolarisation sufficient to induce action potentials. Suprathreshold TMS intensities in humans are typically defined as the stimulus intensity that can elicit an observable or measurable physiological sensation or movement (e.g. a phosphene or MEP), and varies between subjects. The magnetic field intensities which produce suprathreshold stimulation are in the same magnitude as MRI scanners but last less than a millisecond per pulse (Ridding and Rothwell, 2007). Suprathreshold stimulation occurs at the “hot spot” directly underneath the coil windings (or at the intersection of the coils in the figure of 8 designs) whereas subthreshold stimulation occurs in the regions adjacent to the hot spot (Wagner et al., 2009) (figure 1.6).

To date there are multiple methods of reporting rTMS intensity beyond suprathreshold and subthreshold. rTMS intensity is often reported as a percentage of the TMS machine stimulator output (MSO%) making it difficult to interpret as the electromagnetic field produced for stimulation varies between TMS machines/companies and is heavily dependent on the design and specifications of the coil used. rTMS intensity can also be defined as a percentage of an individual’s resting motor threshold (RMT) as assessed by MEP/EMG assessment to standardise the intensity of stimulation across individuals. Both measures become inappropriate when low intensity stimulation is used. It is only when delivering stimulation at frequencies
≤ 300Hz and with magnetic fields of up to a millitesla or less, that the stimulation intensity is reported as the absolute value of the magnetic field rather than as a percentage of the subject’s RMT or MSO. In this case, studies are placed into the “extremely low field magnetic fields” (ELFMF’s) category (Di Lazzaro et al., 2013). This level of quantification and characterisation is more appropriate for experimental (i.e. in vitro) and animal rTMS models, as not all studies are conducted on the motor cortex or in preparations where RMT can be determined. Furthermore, custom made stimulators are often used in these preparations as commercial stimulators are commonly designed for the use in humans and not in small animals or single cells. In this thesis, I report the use of stimulation intensities ≥1mT and <1T and define this range as low intensity rTMS (LI-rTMS), in contrast to ELFMF (<1mT) and HI-rTMS intensities (≥1T).

**Figure 1.6. Induced current distribution with high intensity rTMS.** High intensity rTMS can be targeted to a specific region (red), however adjacent regions also receive low intensity stimulation (green). Therefore rTMS-induced plasticity may be the result from neuromodulation with both high and low intensity stimulation. Figure adapted from (Wagner et al., 2007).

### 1.5 rTMS induced plasticity

Due to the high level of plasticity retained in the motor cortex, investigation of rTMS modulation of the human motor cortex has been the main focus of rTMS plasticity research.
An additional attraction to studying rTMS-induced plasticity in the motor cortex is the simple and non-invasive method of probing any induced changes with single pulse TMS and the induced muscle evoked potential (MEP). Although not directly measured, Barker and colleagues (Barker et al., 1985) demonstrated in their seminal study that a single suprathreshold pulse delivered to the appropriate brain region results in a muscle response in a specified muscle (Barker et al., 1985) which can be measured with surface EMG electrodes and reflect changes in the excitability of the corticospinal tract.

1.5.1 Changes in corticospinal excitability

In humans, rTMS-induced plasticity is believed to be frequency dependent as low and high frequency rTMS commonly result in depression and facilitation of MEP amplitudes respectively (Hoogendam et al., 2010). Frequency dependency is also observed with complex frequencies such as TBS protocols, where cTBS and iTBS produce opposite changes to MEP amplitude with cTBS used to induce MEP amplitude depression and iTBS for MEP amplitude facilitation (Huang et al., 2005).

Although not as well characterised in animals, rTMS can alter corticospinal excitability in rats and is also frequency dependent, as has been shown in humans (Muller et al., 2014, Hsieh et al., 2014). In anaesthetised rats, 1Hz rTMS depresses MEP amplitudes and lasts approximately 30 minutes whereas 0.25 and 0.5Hz rTMS does not alter MEP amplitudes (Muller et al., 2014). Using complex patterned protocols, cTBS and iTBS rTMS in anaesthetised rats results in MEP depression and facilitation respectively (Hsieh et al., 2014). Interestingly, when delivered using direct electrical stimulation as a proxy for magnetic stimulation, cTBS results in synaptic facilitation whereas iTBS results in synaptic depression (Barry et al., 2014).

1.5.2 Change in motor learning and behaviour

The use of non-invasive brain stimulation to enhance motor skills has gained significant interest as it may act as a useful adjunct therapy for neurological disorders (e.g. stroke and Parkinson’s disease) and to promote healthy ageing. Given the extensive characterisation of TMS effects in the motor cortex using MEPs, it is not surprising that
TMS-induced plasticity is commonly investigated using motor behaviour in both clinical and non-clinical populations (Reis et al., 2008).

Early investigation with single pulse TMS to the primary motor cortex simultaneous to the contralateral hand practicing a thumb abduction task showed that TMS could transiently enhance motor memory (Classen et al., 1998). In contrast, single pulse TMS to the ipsilateral motor cortex cancelled the effect of motor training, demonstrating that TMS can be used to modulate motor behaviour. Moreover, it has been suggested that increasing the excitability of the motor cortex undergoing learning, or decreasing the excitability of the resting motor cortex (i.e. motor cortex not undergoing learning), can facilitate motor learning (Reis et al., 2008), making the use of rTMS to facilitate motor learning promising.

One of the earliest demonstrations that rTMS could modulate motor learning delivered 1Hz rTMS to the motor cortex prior to a thumb to finger motor task (Muellbacher et al., 2002). Interestingly, this inhibitory form of rTMS did not alter finger speed or muscle force generation, but had a negative impact on skill retention. Other studies investigating the use of rTMS prior to motor learning (i.e. as a priming stimulus) have produced mixed results. Teo and colleagues used a single session of iTBS prior to motor training on a thumb acceleration task analogous to throwing, and showed that iTBS increased performance variability and the rate of learning (Teo et al., 2011). Jelic and colleagues performed similar experiments with the use of both cTBS and iTBS using a skilled motor task (purdue pegboard task) and measured corticospinal excitability (Jelić et al., 2015). Their results mimicked the seminal work of Huang showing that cTBS decreased cortical excitability whereas iTBS increased cortical excitability. When examining skilled motor behaviour, cTBS slowed motor learning whereas iTBS did not increase motor learning, in contrast to the work of Teo and colleagues. When given for 4 consecutive days prior to mirror training, iTBS over the dorsal premotor cortex resulted in a significantly poorer performance relative to sham stimulated participants (Läppchen et al., 2015), again contradicting the work of Teo and colleagues. A possible explanation is that priming rTMS (an LTP inducing protocol) combined with motor learning (a process underpinned by LTP), may have led to a metaplastic interaction (Läppchen et al., 2015). That is, LTP induced by priming rTMS increased the threshold for LTP and reduced the threshold for LTD, resulting in a
poorer performance. As a result, it was suggested that rTMS should be used with caution for rehabilitation and training in neurological diseases such as stroke.

If rTMS as a priming stimulus interacts with motor learning, can rTMS as a consolidating stimulus enhance motor learning/retention? In an interesting study design, Stockel and colleagues had participants train on a finger abduction task, resulting in increased performance and cortical excitability (Stöckel et al., 2015). Applying iTBS after this training period led to a decline of the increase in motor performance established after training and suggested that iTBS can be used to degrade motor performance.

In summary, the mechanisms underlying both facilitation and depression of motor learning following priming or consolidation rTMS are unclear. In chapter 3 of this thesis, skilled motor learning in mice is combined with chronic rTMS (priming or consolidation) to gain further insight into the potential of rTMS for enhancing motor learning.

1.6 rTMS mechanisms

Despite the use of rTMS to modulate plasticity at the systems level in humans, the cellular and molecular mechanisms underlying rTMS-induced plasticity are unclear. The bulk of the available literature on these basic mechanisms are from animal and experimental models, as changes at the systems level can be further investigated at the cellular and molecular level and have shown the involvement of both synaptic and non-synaptic mechanisms in rTMS-induced plasticity.

1.6.1 Where on the neuron does TMS act?

In both single pulse TMS and rTMS, the induced magnetic field is believed to carry the electrical stimulus across the skull and scalp into the brain (Ridding and Rothwell, 2007). At the time of stimulation, not all neuronal compartments are depolarised equally with the axon believed to undergo the greatest depolarisation (Paulus and Rothwell, 2016), which in turn will induce action potentials if given a large enough stimulus. Axons have a lower action potential threshold relative to the soma (Ridding
and Rothwell, 2007) with the lowest threshold to TMS occurring at curved axonal segments (Tranchina and Nicholson, 1986). However recent evidence has suggested that axons are not the site of TMS activation (Pashut et al., 2011, Pashut et al., 2014). Computer simulations suggest that for neurons with projections extending to a radius smaller than the radius of the magnetic coil, the largest neuronal compartment (soma) undergoes the largest depolarisation leading to action potentials being generated at the axon initial segment. A follow up study from the same lab combined patch clamp electrophysiology of layer 5 pyramidal neurons with suprathreshold magnetic stimulation which provided experimental validation that TMS leads to somatic depolarisation (figure 1.7) prior to action potential induction that occurs proximal to the soma (Pashut et al., 2014).

While the soma is implicated as the main location of the biophysical processes resulting from electromagnetic induction, rTMS induces plasticity that outlasts the time of active stimulation with effects observed in each of the different neuronal compartments, such as the dendrite and synapse and comprises of electrophysiological, structural, molecular and genetic changes (i.e. biological processes) (Gersner et al., 2011, Vlachos et al., 2012, Lenz et al., 2014, Grehl et al., 2015, Lenz et al., 2016).

Contrary to popular belief, Pashut et al suggest that the soma undergoes the greatest depolarisation in response to single pulse TMS. Figure adapted from (Pashut et al., 2011).
1.6.2 Synaptic mechanisms

The mechanisms underlying rTMS-induced plasticity remain unclear, but are believed to involve synaptic plasticity due to the similarities between synaptic plasticity (induced with electrical stimulation) and rTMS induced-plasticity (Hoogendam et al., 2010). Key data supporting synaptic plasticity as a key mechanism of rTMS include: (i) rTMS-induced effects extend beyond the stimulation period, (ii) the frequency and pattern of rTMS pulses rTMS affect the direction and duration of the induced plasticity, (iii) rTMS-induced plasticity interacts with metaplasticity and (iv) rTMS interacts with learning. The most robust evidence describing synaptic mechanisms in rTMS comes from experimental hippocampal slice preparations where the circuit layout and synaptic plasticity mechanisms are well characterised for historical reasons (Bliss and Lømo, 1973). Here I discuss these studies to draw out the synaptic mechanisms of rTMS in normal systems. Additional studies have been conducted in other regions (e.g. cortex and striatum) in the context of disease models and are discussed below as appropriate. When rTMS is applied in vitro, I refer to this procedure as rMS as there is no cranium.

1.6.2.1 Evidence that high frequency rMS induces LTP: in vitro models

In vitro studies of rMS have provided strong support for the synaptic plasticity predicted to occur following human rTMS. High frequency stimulation has been shown in multiple laboratories to induce LTP (Tokay et al., 2009, Vlachos et al., 2012, Lenz et al., 2014). In particular, recent studies by the Vlachos laboratory have provided pivotal insights into possible mechanisms underlying high frequency (10Hz) rMS induced synaptic plasticity at the single cell level using organotypic hippocampal cultures (Vlachos et al., 2012, Lenz et al., 2014). In their first study, the authors demonstrated long-lasting (2-6 hours post stimulation) increases in AMPA receptor mediated synaptic transmission accompanied by remodelling of small dendritic spine populations on CA1 pyramidal neurons (Vlachos et al., 2012). Interestingly, similar to changes seen following classical LTP paradigms, rMS increased AMPA receptor cluster size and number in an NMDA receptor dependent manner.

Their second study demonstrated that increases in the strength of excitatory synapses were predominantly localised to the proximal dendrites and were voltage-gated (sodium and calcium channels), NMDA receptor and calcium dependent (Lenz et al.,
Combing the results of their first two studies, the authors suggest a mechanism behind rMS induced plasticity, which relies on the fact that the induced magnetic field from rMS simultaneously depolarises the pre and post-synaptic neurons (figure 1.8) (Lenz et al., 2014). rMS-induced action potentials in the pre-synaptic neuron result in the release of glutamate into the synapse, while simultaneous depolarisation of the post-synaptic dendrite by rMS activates voltage-gated calcium channels and removes the magnesium block from the NMDA receptor (figure 1.8). This “cooperative” activation of the pre and post-synaptic neurons promotes the accumulation of AMPA receptors on the post-synaptic cell, resulting in an overall strengthening of the synapse and LTP-like effects. Furthermore, the synchronised activation of the pre and post-synapse would account for the induction of LTP-like effects by rTMS frequencies that are much lower than the higher frequency stimulation (100Hz) often needed with direct electrical stimulation in traditional LTP studies.

In their third study, Lenz and colleagues investigated whether the same 10Hz rMS protocol, shown to induce plasticity at the excitatory synapse, would induce plasticity at the inhibitory synapse (Lenz et al., 2016). Their results showed that, similar to the excitatory synapse, 10Hz rMS induced both structural and functional plasticity at the inhibitory synapse. Specifically, rMS decreased GABA\textsubscript{A} receptor mediated synaptic transmission and reduced the number and size of the gephyrin clusters, a post-synaptic scaffolding protein to which GABA\textsubscript{A} receptors anchor (Tyagarajan and Fritschy, 2014). Pharmacological inhibition showed that these rMS-induced changes were voltage gated sodium channel, L-type voltage gated calcium channel and NMDA receptor dependant. Furthermore rMS-induced plasticity was prevented when the downstream calcium signalling pathway (calcineurin protein phosphatases) were blocked.

Interestingly, although gephyrin cluster size and number were reduced with rMS, there was no accompanying change in gephyrin mRNA or protein expression. The authors suggest that rMS gephyrin remodelling is a result of calcium-dependent gephyrin oligomerisation/dissociation rather than a direct modulation on gephyrin synthesis / degradation.

In line with their previous work which showed rMS-induced structural and functional plasticity at the excitatory synapse, rMS-induced structural and functional plasticity at
the inhibitory synapse was localised to the dendritic compartments of the CA1 pyramidal neurons. Possible explanations of why rMS plasticity preferentially occurs at the dendrite and not the soma include differential effects of the electromagnetic field on the somatic vs dendritic compartments, differential intracellular calcium stores and distribution of distinct channels, receptors (subunits) and enzymes and receptor asymmetry, such that inhibitory synapses at the soma are less susceptible to calcium-mediated remodelling. The latter possibly due to the requirement of both NMDA receptor and L type voltage gated calcium channel activation to mediate rMS-induced plasticity not occurring at the soma due to the lack of somatic NMDA receptor synapses (Lenz et al., 2016).

While these studies provide significant insight into high frequency rMS-induced plasticity of subcortical neurons, this has yet to be demonstrated in cortical neurons which have unique properties. Furthermore, whether similar mechanisms are involved in low frequency stimulation and the induction of LTD remains unclear. For example, does rTMS induce LTD via the inhibitory or excitatory synapse and their associated receptors, or both?

Interestingly, it has been proposed that rTMS induces LTD by altering the efficacy of the excitatory synapse, perhaps through altered AMPA receptor trafficking (Thickbroom, 2007), and the hypothesis is consistent with evidence from the above studies by the Vlachos laboratory (i.e. that rMS induced LTP results in increased AMPA receptors at the excitatory synapse). However, histological rat studies have shown rTMS may act on both excitatory and inhibitory neurons (Trippe et al., 2009, Hoppenrath and Funke, 2013).

In addition to inducing synaptic plasticity, rMS has also been shown to modulate the capacity for LTP (i.e. metaplasticity). Tokay and colleagues delivered trains of 100Hz rMS 30 minutes prior to the induction of electrically induced LTP (Tokay et al., 2014) in the well characterised hippocampal slice model (Bliss and Lomo 1973). Unlike control slices which exhibited LTP after electrical stimulation, slices primed with 100Hz rMS exhibited LTD after electrical stimulation. Similar to the studies using rMS to induce
synaptic plasticity, the effects of LTD-like rMS protocols on metaplasticity are not known.

Figure 1.8. “Cooperative” mechanism of rMS-induced LTP at the excitatory synapse in the hippocampus. The proposed mechanism by Vlachos and colleagues suggests rMS induces LTP via simultaneous activation of the pre- and postsynaptic cell and is facilitated through voltage-gated calcium and NMDA receptor dependent calcium influx. Activation of these processes leads to a transient (~2–6 hours post-stimulation) enlargement of specific spine populations and an accumulation of AMPA receptors on the postsynaptic cell. Whether similar processes are involved in the induction of LTD-like plasticity is unknown. LTP = long-term potentiation; rMS = repetitive magnetic stimulation; LTD = long-term depression.

1.6.3 Differential effects on inhibitory and excitatory networks

In humans, evidence for differential effects on inhibitory and excitatory networks is indirect. However, in animal models it is possible to identify specific neuron populations that are activated by rTMS and to determine their contribution to excitatory and inhibitory transmission. An elegant series of experiments has shown rTMS-induced changes in both excitatory and inhibitory cell types and has specifically
highlighted the key role of cortical inhibitory interneurons in TBS-induced plasticity (Aydin Abidin et al., 2008, Trippe et al., 2009, Labedi et al., 2014). Furthermore, these experiments suggest that TBS affects multiple brain regions and induces differential activation of inhibitory interneuron subtypes (fast spiking vs non-fast spiking subtypes). Unsurprisingly, NMDA receptors are deeply involved in these processes, with NMDA blockade weakening rTMS effects (Labedi et al., 2014). An extension of this work showed that post stimulation, iTBS-induced modulation can be defined into 3 distinct phases (Hoppenrath and Funke, 2013): (i) An early phase (10-20 minutes post stimulation) of increased excitatory and inhibitory activity, followed by (ii) a late phase (40-80 minutes post stimulation) of decreased inhibitory activity and (iii) a further late phase (160 minutes post stimulation) of decreased excitatory activity. The early increase in inhibition, as reflected by an increased expression of the pre-synaptic isoform of the GABA-synthesising enzyme GAD65, may be related to outcomes in a human study which showed increased cortical GABA release following cTBS (Stagg et al., 2009). The late decrease in inhibition is indicated by a decrease in the calcium-binding protein parvalbumin, a key protein regulating spike activity (Orduz et al., 2013) and GABA release (Vreugdenhil et al., 2003) in fast-spiking inhibitory interneurons. rTMS-induced changes in the electrical properties of neurons (i.e. membrane properties) beyond synaptic strength changes are not well known. Hoppenrath et al have investigated the electrophysiological changes (non-synaptic) on fast-spiking interneurons in the neocortex of young (postnatal day 26-28), adult (postnatal day 29-38) and old rats (postnatal day 40-62) (Hoppenrath et al., 2016). Rats received 1800 pulses of iTBS and cortical slices were prepared from the somatosensory cortex for electrophysiological characterisation 2 hours post stimulation. iTBS increased neural excitability as shown by increased evoked firing and a more depolarised membrane potential, in adult but not young or older animals. Furthermore, iTBS increased the frequency of synaptic currents and increased the amplitude of excitatory postsynaptic potentials in adult animals. In contrast, iTBS did not alter input resistance, membrane time constant, membrane capacitance or action potential threshold. These results suggest that rTMS alters the electrophysiological properties at the single level that occur outside the synapse (e.g. membrane potential), which may underlie rTMS-induced plasticity at the network level. Whether similar changes occur on cortical excitatory neurons are unknown and will be addressed by aim 4 of this thesis.
1.6.4 Non-synaptic mechanisms

1.6.4.1 Biochemical mechanisms

Regulation of gene and protein expression has become a common outcome measure in animal rTMS studies, with targets ranging from neurotrophic factors to neurotransmitters. Of particular interest is the effect of rTMS on the regulation of BDNF, a potent trophic factor implicated in synaptic plasticity, neuronal survival and regeneration. The role of BDNF in TMS-induced plasticity has gained significant attention in the clinical context because humans with genetic variants of BDNF respond differently to TMS protocols (Cheeran et al., 2008), providing a potential biomarker for tailoring treatment. Although the mechanism underpinning the different response in BDNF variants remains unknown, it seems that BDNF plays a key role in the effects of rTMS: studies in humans show upregulation of BDNF in serum samples following HI-rTMS (Zanardini et al., 2006) and studies in rodents confirm that this upregulation occurs in the brain itself (Müller et al., 2000, Gersner et al., 2011, Castillo-Padilla and Funke, 2015) even when stimulation intensity is very low (Rodger et al., 2012, Makowiecki et al., 2014). However, upregulation is not observed in all brain regions (Gersner et al., 2011, Tang et al., 2015b). Confirming the possibility of using BDNF as a biomarker, levels of BDNF in rats following high and low frequency rTMS depend on the conscious state (awake vs anaesthetised) (Gersner et al., 2011). While low frequency rTMS failed to alter BDNF expression in the awake or anaesthetised animal, high frequency rTMS upregulated BDNF in the awake animal but down regulated BDNF in the anaesthetised animal. Although the study did not investigate a functional correlate of these changes in BDNF, these results not only support frequency dependent effects of rTMS, but suggest that spontaneous neural activity may interact with rTMS to regulate BDNF and modulate plasticity. This complexity emphasizes the need for relevant and carefully controlled animal studies to investigate the downstream targets of the TMS-induced BDNF increase and the specific effects on brain function in order to inform the use of BDNF as a biomarker in human studies.
1.6.4.2 Stem cell effects: Neurogenesis

It is now widely accepted that the production of newly derived neurons extends beyond development and occurs within the adult CNS, albeit at a more limited rate. In the adult, neurogenesis primarily occurs sub-cortically in the dentate gyrus of the hippocampus and cortically in the sub-ventricular zone of the lateral ventricles (Ming and Song, 2011).

Although not extensively studied, rTMS has been shown to modulate neurogenesis in rats and mice with mixed results. In intact rats, chronic daily administration (14 consecutive days) of 25Hz rTMS has been shown to increase neurogenesis in the dentate gyrus (Ueyama et al., 2011). Similarly, 14 days of 1 or 30Hz rTMS increases the number of neural progenitor/stem cells in the sub-ventricular zone of mice (Abbasnia et al., 2015). In contrast, 0.2Hz TMS of the rat cortex with recently transplanted human dental pulp stem cells, results in decreased stem cell survival relative to sham stimulation (Kremer et al., 2016).

The mechanism underlying rTMS-induced neurogenesis is unknown but is suggested to involve rTMS modulation of neurotransmitter and neurotrophic factor release (Cullen and Young, 2016). Furthermore, the functional significance of rTMS-induced neurogenesis is unclear as whether these newly derived cells survive or improve behaviour in an intact system has not been investigated.

1.6.5 rTMS at the systems level

Experimental investigation of rTMS at the systems level has primarily focussed on sensory systems such as the sensorimotor and visual systems that can be probed behaviourally. Studies have consistently revealed increased cortical plasticity following rTMS that is associated with modulation of inhibitory cortical circuits and/or BDNF upregulation in one or more brain regions (Mix et al., 2010, Castillo-Padilla and Funke, 2015, Rodger et al., 2012, Makowiecki et al., 2014).

Similar to humans rTMS can improve learning in animals (Mix et al., 2010). In intact rats, iTBS prior to a sensory discrimination task improved learning, while no effect was seen with cTBS or sham stimulation. Changes at the systems level were accompanied
by changes at the cellular level whereby the authors suggest that iTBS improves learning via a mechanism that encompasses both excitatory pyramidal neurons and inhibitory interneurons (Mix et al., 2010). ITBS is proposed to initially induce hypo-activation of inhibitory interneurons and disinhibition of excitatory pyramidal neurons, which subsequently facilitates LTP of the sensory pyramidal neurons, resulting in improved learning (Funke and Benali, 2011). While these experiments were conducted in intact rats and were not restricted to the motor cortex, they provide evidence for the potential for rTMS to manipulate synaptic mechanisms which results in changes at the systems level (learning facilitation).

Another approach to investigate rTMS and plasticity at the systems level outside motor learning has been to look at early development, when sensory experience guides the establishment of neuronal connections. For example in the visual system, development of cortical circuits can be modulated by the light environment (Hensch, 2005) and depriving young rats of visual input during the critical period, delays maturation of cortical circuits (Berardi et al., 2000). In this context, application of iTBS for five days rescued the effects of light deprivation in rats by increasing BDNF levels and reducing the number of parvalbumin positive fast-spiking interneurons; importantly iTBS restored visual function to control levels (Castillo-Padilla and Funke, 2015).

In another model of abnormal visual system development, ephrin-A⁻/⁻ (knockout) mice, low intensity rTMS (~12mT) induced functionally beneficial structural and molecular plasticity (Rodger et al., 2012, Makowiecki et al., 2014). Ephrin-A⁻/⁻ mice lack key axon guidance cues and as a result, topographic organisation of the primary visual projection is disrupted (Feldheim et al., 2000, Haustead et al., 2008, Wilks et al., 2010). Daily stimulation of these mice for 14 days with an experimental high frequency “biomimetic” patterned stimulation (not currently used in humans) decreased the number of abnormal projections in sub-cortical (Rodger et al., 2012) and cortical visual circuits (Makowiecki et al., 2014). Importantly, visuomotor function in these mice was also restored to normal wild type levels (Haustead et al., 2008, Rodger et al., 2012). In addition, increases in BDNF were observed, similar to the effects of iTBS in the developing rat visual system (Castillo-Padilla and Funke, 2015).
Taken together, studies in the developing and mature visual system suggest that rTMS promotes a plastic brain environment that can interact with and perhaps even re-open the critical period of development. Further investigation of rTMS in such classical animal models of development and plasticity may yield novel insights into the prevention and treatment of neurological conditions associated with abnormal cortical development, such as schizophrenia and autism.

1.6.6 rTMS as a treatment for neurotrauma

The use of rTMS to treat neurotrauma has mainly been studied in stroke models. The use of rTMS after stroke has two therapeutic goals: (1) to act as an adjunct to traditional physical therapies to promote functional recovery by modulating synaptic mechanisms and “priming” neuronal circuitry for learning/LTP and (2) to restore abnormal cortical excitability of the affected or non-affected hemispheres (Ridding and Rothwell, 2007). Abnormalities in cortical excitability are a result of the stroke lesion, and are commonly observed as increased inter-hemispheric inhibitory drive from the contralesional hemisphere (Murase et al., 2004). One of the most compelling animal studies to explore the modulation of intracortical inhibition used direct electrical stimulation at iTBS and cTBS frequencies as a proxy for rTMS in order to retain the focality and intensity used in human treatment (Barry et al., 2014). Importantly, this study first defined the stimulation conditions required to reduce inter-hemispheric inhibition in intact rats and then applied these conditions to rats having undergone a cortical lesion. ITBS, but not cTBS induced synaptic depression in intact animals, thereby down regulating the efficacy of inter-hemispheric inhibitory circuits. ITBS applied to rats with a cortical lesion resulted in reduced inter-hemispheric inhibition and improved motor function. In contrast, cTBS induced facilitation and did not improve function. Interestingly, the outcomes of cTBS and iTBS with electrical stimulation in rats appear to be contradictory to their effect in the normal human brain (Huang et al., 2005), although more recent evidence suggests high variability in human response to different TBS paradigms (Hamada et al., 2013, Hinder et al., 2014).

One unexpected outcome of animal studies of rTMS has been to identify a neuroprotective function. As a result, there is growing interest in the use of brain
stimulation to limit the amount of cell death and thus functional loss. Direct electrical stimulation (Glickstein et al., 2001) and rTMS (Fujiki et al., 2003, Ogiue-Ikeda et al., 2005) delivered prior to the induction of ischemia can decrease cell loss and reduce infarct size. There is evidence suggesting rTMS reduces cell death and functional loss through the up regulation of anti-apoptosis proteins (Yoon et al., 2011). However, increases in anti-apoptotic proteins are not accompanied by changes in markers of neural plasticity (e.g. NMDA receptors), which suggests that neuroprotective mechanisms may be independent of changes in neural plasticity (Yoon et al., 2011). An alternative mechanism that affects both neuroprotection and plasticity is the up regulation of BDNF (Zhang et al., 2007), arguably one of the most commonly observed molecular changes following non-invasive brain stimulation. Alternatively, or in addition to reducing neuronal death, rTMS may increase neurogenesis. A recent study found daily 10 Hz rTMS for 1 week improved neurological function (decreased Neurological Severity Score: assessment of motor, touch, reflex and balance) and increased neural stem cell proliferation in the subventricular zone of adult rats after focal ischemia (Guo et al., 2014). Although neurogenesis is spontaneously increased in rats after a stroke (Arvidsson et al., 2002), chronic 10Hz stimulation significantly increased neural stem cell proliferation (a 3.6 fold increase) compared to non-stimulated animals (Guo et al., 2014). However, whether these newly proliferated stem cells deliver any functional benefit is unknown.

Recent work from our lab has further explored therapeutic rTMS mechanisms outside modulation of cortical excitability by investigating potential neuronal regeneration following partial CNS injury. In cerebellum explants, unilateral lesion to inferior olive results in the degeneration of the respective cerebellar climbing fibre tract and denervation of the contralateral cerebellar hemisphere (Bower and Sherrard, 1986). Using low intensity rMS with a protocol shown to induce molecular and structural plasticity in vivo (Rodger et al., 2012, Makowiecki et al., 2014), we showed that 14 days of rMS to the cerebellum induces collateral sprouting from the cerebellar hemisphere ipsilateral to the lesion, resulting in partial re-innervation of the denervated cerebellar hemisphere (Morellini et al., 2014). However, whether neuronal regeneration could be induced with rTMS in a complete CNS injury is unknown.
1.6.7 High intensity vs low intensity rTMS-induced plasticity mechanisms

For the most part, rTMS studies have focussed on the use of HI-rTMS with even less known about the effects of LI-rTMS on neural plasticity. The few studies that have been done have shown that subthreshold currents induced by LI-rTMS are capable of inducing plasticity (i.e. action potential independent plasticity) (Makowiecki et al., 2014, Rodger et al., 2012, Capone et al., 2009). Furthermore, as LI-rTMS does not produce MEPs or phosphenes or modulate their characteristics, it is assumed that LI-rTMS is not clinically relevant. This may be an inappropriate assumption as the poorly understood cellular changes after LI-rTMS/subthreshold stimulation may contribute significantly to the overall plasticity induced with rTMS, which is assumed to have been induced solely by HI-rTMS. Similarly, whilst MEP amplitudes and resting motor threshold are easily determined and modulated in humans with high intensity TMS, they are not translatable beyond the motor cortex and provide limited information on the cellular and molecular mechanisms induced by TMS relative to animals and experimental models.

Plasticity induced by HI-rTMS and LI-rTMS may be underpinned by a mixture of overlapping and distinct mechanisms. Recent in-vitro work from our lab has shown that low intensity repetitive magnetic stimulation (LI-rMS) (13mT) increases intracellular calcium concentrations (Grehl et al., 2015). Whilst increased intracellular calcium is believed to occur with HI-rTMS (Lenz et al., 2014), this calcium influx was from intracellular stores and not from the extracellular milieu (i.e. not due to action potentials induced by HI-rTMS). A potential overlapping mechanism between LI-rTMS and HI-rTMS is BDNF. In awake mice, high frequency LI-rTMS increases BDNF in the visual cortex and superior colliculus (Rodger et al., 2012, Makowiecki et al., 2014). Similarly, high frequency HI-rTMS delivered to awake rats increases BDNF in the hippocampus and prelimbic cortex (Gersner et al., 2011).

**Aims**

Despite the wide use of rTMS in both clinical and non-clinical populations, there is a great need to better understand the basic physiological mechanisms underlying rTMS to fully exploit its neuromodulatory potential (Wassermann and Zimmermann, 2012).
This thesis aims to investigate the mechanisms underlying LI-rTMS-induced plasticity behaviour in healthy and neurotrauma rodent and experimental models (i.e. ex vivo brain slices). Rodent and experimental models were chosen due to the more direct and advanced methods available to probe plasticity in addition to the behavioural paradigms available. Studies investigating the use of LI-rTMS in humans are the focus of other studies in our lab and are not discussed here.

The lack of current animal and experimental rTMS data is attributed to the lack of appropriate stimulator equipment available to focally stimulate rodent brains and experimental tissue (Wassermann and Zimmermann, 2012). The studies in this thesis aimed to address this limitation through the use of low-intensity (12mT) rodent-specific rTMS coils (Rodger et al., 2012, Makowiecki et al., 2014) and by designing and constructing a novel rodent-specific rTMS coil to deliver moderate field strengths (~100mT).

**Aim 1: rTMS as a potential therapy for neuroprotection and axonal regeneration**

Unlike the peripheral nervous system (PNS), the CNS does not regenerate after injury and trauma and often leads to severe functional impairment. As a result, there have been great efforts to develop treatments and therapies that can overcome the degenerating mechanisms that follow neurotrauma. The optic nerve is an attractive model to investigate neural regeneration and cell survival as it is a white matter tract that consists of axons from a single cell type, the retinal ganglion cells (RGC’s) (Benowitz et al., 2015). RGC axons enter the optic nerve from the retina and project to cortical and sub-cortical regions of the brain. Crushing the optic nerve severs the RGC axons without severing the optic nerve sheath and depending on the duration of the crush, does not rupture blood vessels or lead to concurrent ischaemic injury (Tang et al., 2011). Severing of RGC axons does not lead to instant RGC death (i.e. at the time of injury) but is delayed to approximately 5 days after injury and depending on the severity of the injury, can result in less than 10% RGC survival at two weeks post injury (Berkelaar et al., 1994). Therefore, optic nerve repair strategies must be two fold such that they promote RGC cell survival (i.e. neuroprotection) in order for axonal outgrowth (i.e. regeneration) to occur.

One common strategy to induce neuroprotection is through the use of neurotrophins. Following an optic nerve crush, RGC cell bodies become disconnected from their...
central target which prevents the retrograde transport of neurotrophins from the
cortex to the retina which is suggested to trigger RGC death (Fischer and Leibinger,
2012). Of the neurotrophin family, BDNF is the most widely studied neurotrophin for
optic nerve repair due to its potent neuroprotective effect on RGCs (Almasieh et al.,
2012). The neuroprotective effect of BDNF on RGCs was first characterised in vitro,
where embryonic rat RGCs co-cultured with BDNF had greater survival rates (Johnson
et al., 1986). In vivo, intravitreal injections of BDNF delays RGC death by promoting
RGC survival following optic nerve injury in rodents and cats (Mansour-Robaey et al.,
cats, single intravitreal injections of varying BDNF concentrations of BDNF (15, 30, 60,
and 90 mg) increased RGC survival after an optic nerve crush, with 30mg having the
greatest effect (81% RGC survival vs 49% in sham) (Chen and Weber, 2001). In a follow
up study, Weber and colleagues showed that a single dose of BDNF induced RGC cell
survival which was maintained at 2 weeks post injury (1 week= 79% BDNF vs 55%
control, 2 weeks= 60 vs 31%) (Weber et al., 2010). In addition to neuroprotection,
BDNF has also been implicated in axonal sprouting (Mansour-Robaey et al., 1994) and
decreased axonal die back (Weibel et al., 1995), making it an attractive treatment for
optic nerve injury.

An alternative to direct treatment with BDNF is the use of electrical stimulation
methods to induce neuroprotection and regeneration. In optic nerve transection
rodent models, electrical stimulation has produced mixed results in preventing RGC
death. Early studies using optic nerve transections and electrical stimulation of the
optic nerve stump (Morimoto et al., 2002) and of the cornea (transcorneal electrical
stimulation; tES) (Morimoto et al., 2005) showed decreased RGC cell death following
stimulation. A single one hour session of varying current intensities (all at 20Hz)
electrical stimulation showed an increase in RGC survival one week following injury
compared to sham stimulation (54% RGC survival) (Morimoto et al., 2002). Increasing
stimulus intensity leads to a greater increase in RGC survival in the lower range of
stimulus intensity (20µA=64%, 30µA=76%, 50µA=83%) but decreased after 50µA with
higher intensities (80µA and 100µA) leading to detached retinas (albeit in a small
sample size), suggesting that high intensity stimulation may not be appropriate for
optic nerve repair. Interestingly, a follow up study from the same lab using a single
session of 100µA of varying stimulus pulse duration (0 to 3ms) and showed that greater pulse duration lead to the greater amount of RGC survival (3ms=70%, sham=54%). Tagami and colleagues extended this by investigating the number of daily tES stimulation sessions (1, 2, 4 and 12 sessions) with 20Hz 100µA on RGC survival and axonal regeneration (Tagami et al., 2009). They showed that increasing the number of daily tES stimulations lead to greater RGC survival as well as increased number of regenerating axons in the optic nerve following an optic nerve crush. In contrast, Henrich-Noack and colleagues have shown that 100µA tES at 20Hz leads to a transient increase in RGC survival (3 days post optic nerve crush) which is not observed at 7 and 15 days post injury. Similarly, the use of transcorneal alternating current stimulation (tACS), has been shown to induce neuroprotection following optic nerve crush (28.2% tACS, 8.2% sham) but does result in any functional benefit (Henrich-Noack et al., 2013a). However, to our knowledge, the use of magnetic fields to induce neuroprotection and axonal regeneration in the optic nerve has not been investigated.

Chapter 2 will investigate the effect of a low-intensity rTMS in a complete optic nerve crush model. The frequency (biomimetic, see chapter 2 for details) and intensity (12mT) was chosen as previous studies from our lab have used this protocol to upregulate BDNF in the mouse visual system (Rodger et al., 2012, Makowiecki et al., 2014) and induce axonal outgrowth in the form of collateral sprouting in a partial CNS injury (Morellini et al., 2014). Retinal ganglion cell survival and axon regeneration will be assessed with immunohistochemistry and changes to BDNF will be assessed with enzyme linked immunosorbent assays.

**Aim 2: Development of higher intensity rodent-specific rTMS coils**

To date, human TMS coils are commonly used in rodent rTMS models, despite the large electromagnetic field that is induced (discussed above). Currently, there is only one commercial animal coil available (Cool-40 Rat coil by Magventure), despite being capable of suprathreshold stimulation (as evident by MEPs), this coil has poor focality and stimulates the entire rat brain and part of the spinal cord (Parthoens et al., 2016).
Chapter 3 will characterise the physical and neuromodulatory properties of two novel rodent-specific rTMS coils capable of delivering stable and consistent moderate intensity (120mT) rTMS. Physical measurements of the magnetic field and sound emissions will be made in addition to finite element modelling of the induced electric field in a simplified rat brain model (Andrea Lowe, University of South Florida). Similar to human rTMS studies in the motor cortex, MEPs can be used to assess rTMS-induced plasticity in rats (Rotenberg et al., 2010, Muller et al., 2014, Hsieh et al., 2014) with suprathreshold TMS pulses used to induce MEPs in the forepaw. Therefore potential neuromodulatory effects will be assessed with single pulse TMS and EMG to investigate changes in MEP amplitudes following rTMS with the novel coils to the rat motor cortex.

**Aim 3: Characterisation of rTMS on skilled motor learning**

rTMS is commonly used to modulate motor learning in humans (Reis et al., 2008). This has gained therapeutic interest as a possible adjunct treatment for neurological conditions where skilled motor function is impaired (e.g. post-stroke) and for assisting with healthy ageing in older adults. rTMS studies in humans have produced mixed results of the efficacy of rTMS to facilitate motor learning (see changes in motor learning and behaviour above) with little known on the mechanisms by which rTMS promotes or impairs motor learning. Furthermore whether rTMS should be given as a priming (before motor training) or consolidating (after motor training) stimulus is unclear.

Chapter 4 will investigate whether chronic moderate-intensity rTMS can consolidate or prime skilled motor learning in adult mice. Changes in skilled motor learning will be assessed with 10 consecutive days of skilled motor learning behavioural testing combined with rTMS (separate priming and consolidation rTMS groups). Potential changes to key synaptic proteins and BDNF expression will be assessed with ELISA and western blots respectively.
**Aim 4: Investigation of rTMS on neural excitability at the single cell level**

rTMS changes in neural excitability are often investigated at the network level through changes in MEP in humans and rats. Recent experimental models using organotypic hippocampal tissue cultures (Vlachos et al., 2012, Lenz et al., 2014, Lenz et al., 2016) (see synaptic mechanisms above) and brain slices (Tokay et al., 2009, Hoppenrath et al., 2016) have expanded our knowledge of the changes in neuronal electrophysiology beyond changes in MEPs. These models are useful as they allow for the assessment of neural excitability at the network and single cell level and can be used to investigate electrophysiological changes of specific neuron subtypes in specific networks. Moreover, the effect of rMS on the electrical properties on individual fast-spiking interneurons 2 hours post-stimulation has recently been examined (Hoppenrath et al., 2016). However, whether rMS alters the electrical properties of excitatory neurons at the single cell level and whether such changes occur immediately after stimulation (i.e. in the first 20 minutes) where changes in MEPs are observed (Hsieh et al., 2014) are unknown.

Chapter 5 will investigate the acute effects of rMS on electrophysiological properties at the single cell level using mouse cortical layer 5 pyramidal neurons (the primary output neurons of the cortex) *in vitro*. Passive and active membrane properties will be characterised with whole-cell patch clamp recordings immediately after stimulation, 10 minutes post-stimulation and 20 minutes post-stimulation.
Chapter 2: Low intensity repetitive transcranial magnetic stimulation does not induce cell survival or regeneration in a mouse optic nerve crush model


**Introduction**

Non-invasive brain stimulation can be used to modulate neural activity in the central (CNS) and peripheral nervous systems (PNS) and has been applied in diagnosis and treatment of neurological disorders. One form of non-invasive brain stimulation is repetitive transcranial magnetic stimulation (rTMS), in which time-varying magnetic pulses from a coil placed over the skull induce electrical currents in the underlying brain by Faraday Induction. rTMS is used clinically at high and low intensities in a wide range of neurological and psychiatric conditions, with therapeutic effects that can persist for hours to days after stimulation (Kleinjung et al., 2005, Bortolomasi et al., 2007, Khedr et al., 2010, Di Lazzaro et al., 2013).

The best-characterised effects of rTMS in human patients are alterations in cortical excitability that persist beyond the time of stimulation (Huang et al., 2005, Peinemann et al., 2004). Mechanisms underpinning these effects have been explored in animal models and demonstrate altered synaptic plasticity in the form of long-term potentiation (Vlachos et al., 2012, Lenz et al., 2014). Furthermore, functional imaging of human patients suggests that repeated rTMS delivery may trigger structural and functional reorganisation (May et al., 2007) and our recent work in mice has confirmed structural and functional reorganisation of abnormal brain circuits via removal or shifting of inappropriate connections, even using low intensity magnetic stimulation (LI-rTMS) (12mT field strength) (Rodger et al., 2012, Makowiecki et al., 2014).
Whilst the biological mechanisms of rTMS are poorly defined, a key molecule upregulated by both rTMS and LI-rTMS effects is brain derived neurotrophic factor (BDNF) (Müller et al., 2000, Gersner et al., 2011, Makowiecki et al., 2014, Rodger et al., 2012), a powerful and versatile signalling molecule that plays many roles not only in synaptic plasticity, but also in promoting neuronal survival and axonal outgrowth. Furthermore, delivery of exogenous BDNF either by viral overexpression or injection of recombinant protein showed neuroprotective and neuroregenerative effects in a range of CNS injury models (Isenmann et al., 1998, Dixon and Sherrard, 2006, Koda et al., 2004). We thus hypothesised that LI-rTMS may be useful in promoting cell survival and/or axonal regeneration following brain injury, via up-regulation of BDNF. In agreement with this hypothesis, there is some indication that rTMS may promote neuronal survival in the lesion site following an ischaemic stroke (Gao et al., 2010), and studies in the PNS show that direct electrical stimulation can promote regeneration following nerve damage (Weibel et al., 1995, Templeton and Geisert, 2012). However, the use of rTMS as a neuroprotective and/or neuroregenerative intervention following neurotrauma has not been well characterised.

Here we investigate the effects of LI-rTMS on neuronal survival and axonal regeneration using a complete optic nerve crush model. The optic nerve is a white matter tract, consisting of axons from a single cell type in the retina, the retinal ganglion cell (RGC). The absence of any surrounding gray matter allows for the investigation of cell survival and neuronal regeneration as distinct events following injury (Templeton and Geisert, 2012). In addition, optic nerve injury models have been used extensively to investigate the potential of both neurotrophin (Templeton and Geisert, 2012, Chen and Weber, 2001) and electrical stimulation treatments on cell survival and regeneration (Henrich-Noack et al., 2013a, Henrich-Noack et al., 2013b, Morimoto et al., 2002, Morimoto et al., 2005). Therefore the optic nerve crush provides an ideal model to investigate the efficacy of LI-rTMS in neuroprotection and regeneration. We delivered LI-rTMS for 10 minutes daily for 14 days at a high frequency biomimetic pattern to the left eye of C57Bl/6J mice starting from the day after optic nerve crush. We chose this protocol because we have previously shown that it induces plastic reorganisation and robust up-regulation of BDNF expression in
the mouse visual cortex, superior colliculus (Rodger et al., 2012, Makowiecki et al., 2014) and cerebellum (Morellini et al., 2014). Here, we found that LI-rTMS had no effect on RGC survival or axonal regeneration, and that BDNF expression was not up regulated in the retina or optic nerve of non-injured and injured mice (optic nerve crush). Our findings suggest that different brain regions may respond differently to LI-rTMS in terms of BDNF up-regulation, and that the therapeutic applications of LI-rTMS will need to be tested in a range of models in order to establish the scope and limitations of this technique.

Methods

Animals

3 month old C57Bl/6J mice (Mus Musculus) (n=34) (Animal Research Centre, Murdoch University, WA, Australia) were group housed on a 12-hour light/dark cycle with food and water ad libitum. All animal work was conducted according to Australian and international guidelines. Mice were euthanased with an overdose of pentobarbitone (>160mg/kg i.p.) and anaesthetised with ketamine and medetomidine (75 and 1 mg/kg respectively). Procedures were approved by the Animal Ethics Committee of the University of Western Australia (approval id: RA100/03/1214).

Optic nerve crush

Surgery was performed on the left eye to obtain a complete unilateral optic nerve crush. Mice were anaesthetised with an intraperitoneal injection of ketamine and medetomidine (75 and 1 mg/kg respectively, Troy Ilium, NSW, Australia). An incision to the lateral conjunctiva allowed for slight rotation of the globe. Muscle and connective tissue were gently separated to expose the optic nerve. The exposed nerve was crushed using Dumont #5 forceps (World Precision Instruments, FL, USA) for 5 seconds, 2-3mm from the optic nerve head. Forceps were gently removed, allowing
the eye to rotate back into place. Anaesthesia was reversed with subcutaneous injection of atipamezole (1 mg/kg, Troy Ilium, NSW, Australia).

The contralateral (non-injured) retina was not used for control tissue, as unilateral optic nerve crushes can induce bilateral glial cell activation (Bodeutsch et al., 1999, Panagis et al., 2005) and RGC loss (Macharadze et al., 2009, Liu et al., 2014).

**LI-rTMS**

LI-rTMS or sham (handling control without stimulation) was delivered daily to the operated eye for 10 minutes. Mice were randomised into two cohorts (see figure 2.1). In cohort 1 (n=14), mice received an optic nerve crush and 14 days of stimulation (sham n=6, LI-rTMS n=8) to assess RGC survival and axonal regeneration. In cohort 2 (n=20), mice received 7 days of stimulation to assess changes in BDNF concentrations with or without an optic nerve crush. Such that there were 5 animals per group (LI-rTMS + optic nerve crush, sham + optic nerve crush, LI-rTMS without optic nerve crush and sham without optic nerve crush). An electromagnetic pulse generator (Global Energy Medicine, WA, Australia) delivered a high frequency complex pattern of stimulation consisting of 59.9ms trains of 20 pulses with trains repeated at 6.67Hz for the first minute, 10.01Hz for 8 minutes and then 6.25Hz for 1 minute. This protocol was selected as it mimics endogenous patterns of electrical activity in the nervous system (patent PCT/AU 2007/00045) and has been shown to up-regulate BDNF and facilitate circuit reorganisation in the visual system (Rodger et al., 2012, Makowiecki et al., 2014) and cerebellum (Morellini et al., 2014).

A custom-made coil (8mm outer diameter, consisting of 300 windings of 0.25mm copper wire, with a steel bolt core, 16Ω) was used to deliver LI-rTMS (Rodger et al., 2012) to the operated eye. Magnetic field strength was measured with a Hall Effect probe (Honeywell SS94A2D, USA). Magnetic field strength decreased with distance from 12mT at the base of the coil to 1.8mT at a distance of 8mm from the coil base. Therefore we estimate the magnetic field strength in the orbital area ranged from 7.4 to 1.8mT. Conscious animals were stimulated under light manual restraint, to avoid possible confounding effects of anaesthetic as opposing effects of rTMS have been observed under anaesthesia (i.e. rTMS down-regulates BDNF in anaesthetised animals).
(Gersner et al., 2011). Animals were placed head first into a small clear conical cylinder with a breathing hole at the end and were habituated to handling and restraint for a week prior to experimentation. Stimulation was delivered through the plastic cylinder, such that the coil was placed directly on the cylinder, immediately over the eye. We have previously shown the coils do not generate vibration (Grehl et al., 2015) and the plastic does not impede the magnetic field.

**Tissue preparation**

24 hours after the last stimulation, mice were terminally anaesthetised with 160mg/kg pentabarbitone sodium and transcardially perfused with 0.9% saline followed by 100mL of 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.2). The left eye with optic nerve was dissected and separated at the optic head. Left eyes were enucleated and whole retinas dissected from the sclera. Retinas were post fixed for 12 hours in fixative solution and stored in PBS with 0.01% sodium azide prior to immunohistochemical analysis. Optic nerves were post fixed in fixative solution and transferred into 30% sucrose in PBS at 4°C for 48 hours. Optic nerves were embedded in optimum cutting temperature medium (Sakura, OH, USA) at -20°C. A Leica CM1900 cryostat was used to cut 14μm transverse sections that were thaw mounted onto gelatin coated glass slides for immunohistochemical analysis.

**Immunohistochemistry**

RGC survival and axonal growth was evaluated in the retina and optic nerve respectively. Retinas were processed free-floating and permeabilised with 0.2% Triton in 0.1M PBS followed by blocking with 10% donkey serum in 0.2% bovine serum albumin (Sigma Aldrich, MO, USA) in 0.1M PBS. Retinas were incubated with for β-3 tubulin primary antibody (1:1000 monoclonal mouse) (Merck Millipore, VIC, Australia) at 4°C for 24 hours. Following 3 rinses in PBS, retinas were incubated with Alexafluor-488 donkey anti-mouse IgG (Life Technologies, VIC, Australia) at room temperature for
4 hours. After three 10 minute washes in PBS, retinas were flattened onto glass slides and cover-slipped with Fluoromount-G (Sigma Aldrich, MO, USA).

Axonal regrowth of RGC axons was assessed in the optic nerve using growth associated protein 43 (GAP43) immunohistochemistry (de Lima et al., 2012, Yin et al., 2003). Optic nerve sections were permeabilised and blocked as described above and incubated with GAP43 primary antibody (1:1000 monoclonal mouse) (Merck Millipore, VIC, Australia) at 4°C for 24 hours. Following washes in PBS, optic nerve sections were incubated with Alexafluor-488 donkey anti-mouse IgG at room temperature for 4 hours, washed with PBS and cover-slipped as described above. GAP43 immunofluorescence was analysed with fluorescence microscopy (Nikon Eclipse 80i, 40 x objective) (LMG Scientific Services, WA, Australia).

**Stereological analysis of retinal wholemounts**

Retinal wholemounts were analysed with the optical fractionator method to estimate the number of β-3 tubulin positive RGCs (Coimbra et al., 2009). Wholemount outlines were digitised on a microscope (Olympus BX50, 10x objective), equipped with a motorised stage, and analysed with Stereoinvestigator software (MicroBrightField, VT, USA) (20x objective). RGCs were counted in a 150x150µm frame. Counting frames were placed systematically within a grid to achieve approximately 200 counting sites, covering approximately 25% of the total area of each retina. Extrapolated RGC populations were divided by retinal wholemount area and remaining RGCs (survival) expressed as RGC/mm².

**Enzyme-Linked Immunosorbent Assay (ELISA) and Protein Assay**

24 hours after the last stimulation, retinas and optic nerves were dissected from freshly euthanised mice (crush + sham, crush + LI-rTMS, non-injured + sham, non-injured + LI-rTMS) and stored at -80°C. Samples were homogenised in 1mL of lysis buffer (Szapacs et al., 2004) (100 mM PIPES pH 7, 500 mM NaCl, 0.2% Triton X-100,
2mM EDTA) with mini protease inhibitor tablets (Roche Biochemicals, IN, USA)(1 tablet added per 10ml buffer). Lysates were centrifuged (3320 x g at 4°C for 1 hour) to collect resulting supernatants. Supernatants were analysed by ELISA for BDNF as per manufacturer’s instructions (ChemiKine™ BDNF Sandwich ELISA, Chemicon International Inc., CA, USA). In addition, supernatants were analysed for total protein content (Pierce® BCA Protein Assay Kit, Thermo Fisher Scientific©, IL, USA, as per manufacturer’s instructions). Supernatant BDNF concentrations were normalised to supernatant total protein content for analyses and expressed as a percentage of the sham group for analyses (figure 2.3).

**Statistical Analysis**

Statistical analyses were performed with SPSS® (version 20, IBM, NY, USA). Normal distribution and homogeneity of variance were verified before running parametric analyses. All means are presented with their respective standard error of the mean (i.e. mean ± SEM). Analyses were conducted on RGC survival (unpaired t-test) and BDNF concentrations (two-way between subjects ANOVA with Sidak corrected post-hoc tests). Results were classified as significant if p<0.05.

**Figure 2.1. Diagrammatic representation of the study design.** Mice received an optic nerve crush and were separated into two cohorts for (i) daily LI-rTMS or sham stimulation and assessed for RGC survival and axonal regeneration (2 weeks survival) (ii) quantification of BDNF levels by ELISA (1 week survival). With the second cohort, additional control groups of intact mice with no optic nerve crush were processed in parallel for BDNF analysis with the same LI-rTMS or sham stimulation parameters.
Results

**LI-rTMS does not increase RGC survival**

To investigate whether 14 days of LI-rTMS could induce neuroprotection and cell survival, we characterised the number of surviving RGCs at 15 days post optic nerve crush injury (Table 2.1). Normal intact C57Bl/6J mice have roughly 4500 RGCs per mm² and cell counts suggest fewer than 10% of RGCs survived the optic nerve crush, with no significant difference in remaining RGCs between sham and LI-rTMS (t = (12)=1.12, p = 0.284).

**Table 2.1 RGC survival and BDNF concentrations following stimulation.**

<table>
<thead>
<tr>
<th>Group + stimulation</th>
<th>Tissue</th>
<th>RGC/mm²</th>
<th>BDNF % of total protein (x10⁻⁵)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>non-injured + sham</td>
<td>Retina</td>
<td>≈4500 (Templeton et al., 2009)</td>
<td>1.26±0.05</td>
</tr>
<tr>
<td></td>
<td>Optic Nerve</td>
<td>-</td>
<td>2.28±0.23</td>
</tr>
<tr>
<td>non-injured + LI-rTMS</td>
<td>Retina</td>
<td>-</td>
<td>1.34±0.01</td>
</tr>
<tr>
<td></td>
<td>Optic Nerve</td>
<td>-</td>
<td>2.80±0.33</td>
</tr>
<tr>
<td>optic nerve crush + sham</td>
<td>Retina</td>
<td>237.2±60.33</td>
<td>1.37±0.088</td>
</tr>
<tr>
<td></td>
<td>Optic Nerve</td>
<td>-</td>
<td>2.97±0.34</td>
</tr>
<tr>
<td>optic nerve crush + LI-rTMS</td>
<td>Retina</td>
<td>335.4±60.74</td>
<td>1.53±0.12</td>
</tr>
<tr>
<td></td>
<td>Optic Nerve</td>
<td>-</td>
<td>2.72±0.29</td>
</tr>
</tbody>
</table>

Mean (±SEM) RGC survival and BDNF concentrations following sham or LI-rTMS. NOTE: RGC survival was quantified following 14 days of stimulation and BDNF was quantified following 7 days of stimulation (*).

**LI-rTMS does not induce RGC axon regeneration**

To assess whether any regenerating RGC axons were present, transverse sections of the optic nerves were labelled using GAP43 immunohistochemistry (Figure 2.2). No
GAP43 labelling was present on either side of the crush site in sham or LI-rTMS groups, indicating no axon regeneration distal or proximal to the crush site.

**Figure 2.2. LI-rTMS does not affect RGC survival or axonal regeneration following optic nerve crush.** A: photomicrograph showing RGCs immunolabelled with β3 tubulin following an optic nerve crush and 2 weeks of daily LI-rTMS. Scale bar is 100 µm. B: Histogram showing counts of surviving RGCs in LI-rTMS and sham stimulated retinas 2 weeks following an optic nerve crush. There was no significant difference between the stimulation groups (p=0.256). Error bars are standard error of the mean. C, D: GAP-43 immunohistochemistry in the proximal (C) and distal (D) optic nerve did not result in labelling of any axons. The crush site is indicated by *. Scale bar 100 µm

**LI-rTMS does not increase BDNF in the retina or optic nerve**

To investigate whether LI-rTMS up-regulates BDNF in intact and injured mice, retinas and optic nerves were collected for BDNF ELISA analysis (Table 2.1 and Figure 2.3). For retinal tissue, there was no significant difference between stimulation condition (sham vs. LI-rTMS) (F[1,16]=1.919  p=0.185) or between injury groups (non-injured vs.
injured) (F[1,16]=2.680, p=0.121). Furthermore, there was no significant interaction between stimulation and injury conditions (F[1,16]=0.209, p=0.653). As we hypothesised a priori that LI-rTMS would up-regulate BDNF compared to sham, despite the non-significant main effect, we conducted Sidak-corrected post-hocs, restricted to comparisons between LI-rTMS and sham, which confirmed mean retinal BDNF concentrations were not significantly different between sham and LI-rTMS treated animals in either the non-injured (p=0.771) and injured (p=0.377) groups.

Similarly, in optic nerve tissue, there was no significant difference between stimulation conditions (F[1,16]=0.199, p=0.661) or injury groups (F[1,16]=1.056, p=0.319). There was no significant interaction between stimulation and injury conditions (F[1,16]=1.626, p=0.220). Follow up Sidak-corrected post-hocs confirmed no significant difference in the mean optic nerve BDNF concentrations between sham and LI-rTMS treated animals in the non-injured (p=0.424) and injured (p=0.820) groups.
Figure 2.3. LI-rTMS does not modulate BDNF. Daily LI-rTMS for 1 week does not increase BDNF levels in the retina or optic nerve of optic nerve crush or intact mice (p>0.05 for all groups; see results section). Histograms show BDNF levels as % of sham stimulated group. Error bars are standard error of the mean.

Discussion

Non-invasive brain stimulation techniques, and in particular rTMS, have become an increasingly common experimental treatment for neurological and psychiatric disorders. However, the extent to which rTMS can be used in the treatment of neurotrauma has not been well characterised. Our results show that, unlike in other visual brain regions examined previously (visual system: (Rodger et al., 2012, Makowiecki et al., 2014); cerebellum (Morellini et al., 2014)), LI-rTMS did not up-regulate BDNF levels in either the intact or injured retina or in the optic nerve, and this was associated with a lack of pro-survival or pro-regenerative effects following injury. The implication is that different brain regions respond differently to LI-rTMS and it will be important to characterise these specific cellular and molecular responses in order to determine relevant and optimised use of electromagnetic stimulation for neural repair.

Does intensity matter?

Typically, rTMS is delivered with commercial human sized coils using high intensity field strengths (≥1T) in both human and animal studies. However, in small animal studies, particularly rodents, a large coil to brain size ratio results in stimulation of the entire brain, if not the whole animal, reducing efficiency of the magnetic field (Weissman et al., 1992). This study delivered LI-rTMS through a custom coil (8mm outer diameter) which allowed for greater focality (Rodger et al., 2012) at the expense of intensity (12mT: approximately 3 orders of magnitude lower than clinical intensities). Therefore, although our results suggest LI-rTMS does not promote cell survival or regeneration, it is possible that high intensity stimulation may have more powerful effects.
The impact of stimulation intensity on neural repair can be directly observed in two studies using rTMS or LI-rTMS in the same rat model of ischaemic stroke. One week of stimulation using a human coil resulted in a significant decrease in the lesion size after ischaemic stroke and improvement in motor behaviour (Gao et al., 2010). By contrast, stimulation with LI-rTMS at the same frequency, although for a shorter duration, did not result in similar benefits (Bates et al., 2012). The importance of stimulation intensity was further highlighted in studies of direct electrical stimulation, in which stimulation with 30 to 70µA, but not 20µA increased RGC survival following an optic nerve transection (Morimoto et al., 2002). Consistent with the different outcomes, there is evidence that rTMS and LI-rTMS activate different mechanisms: HI-rTMS elicits activation of neural circuits via synaptic plasticity (Lenz et al., 2014, Hoogendam et al., 2010) whereas LI-rTMS is subthreshold and exerts its effects by altering membrane potential and neuronal intracellular calcium concentrations without eliciting action potentials (Grehl et al., 2015). Therefore it may be that lower intensities are sufficient to promote circuit reorganisation in intact tissue but higher intensities are required for neuroprotection.

**LI-rTMS effects may be brain region specific**

The cellular mechanisms activated by rTMS and LI-rTMS are poorly understood, but one factor that is commonly detected is up-regulation of BDNF, regardless of the intensity of stimulation (Müller et al., 2000, Zanardini et al., 2006, Gersner et al., 2011, Rodger et al., 2012, Makowiecki et al., 2014). Our previous work showed that LI-rTMS using the protocol applied here induces structural plasticity and up-regulates BDNF in multiple visual brain centres (Rodger et al., 2012, Makowiecki et al., 2014) and in the lesioned olivocerebellar pathway (Morellini et al., 2014). However, in the present study, the same LI-rTMS protocol delivered directly to the eye did not significantly alter BDNF in the retina or optic nerve following 7 days of stimulation. We chose this time-point because of the dynamics of RGC death in our model. Following optic nerve crush, approximately 50% of RGCs survive at one week (Henrich-Noack et al., 2013a, Nadal-
Nicolás et al., 2009), whereas less than 10% remained at 2 weeks. Measuring BDNF at 1 week therefore maximises the chances of detecting changes because the low survival rate at 2 weeks makes a delayed up-regulation unlikely. Nonetheless, we cannot exclude the possibility that up-regulation might have occurred before or after this time.

A possible explanation for the lack of BDNF up-regulation is that the rapid death of RGCs and/or complete discontinuity between the retina and brain targets in our model prevented the effects of LI-rTMS. Supporting this possibility, transcranial alternating current stimulation (tACS) induced EEG after-effects in intact rats but not in animals with severe optic nerve damage (Sergeeva et al., 2012) and the authors suggested that their finding of 9% RGC survival following optic nerve damage was below the threshold needed for tACS to have an effect. A similar problem may apply to our complete optic nerve crush model (<10% RGC survival), whereby LI-rTMS failed to induce positive effects due to too few surviving RGCs and the lack of connections to central targets. This hypothesis is in agreement with our previous studies showing that LI-rTMS promotes beneficial reorganisation of existing connections (Makowiecki et al., 2014, Morellini et al., 2014). Therefore less severe neurotrauma models may respond more effectively to LI-rTMS. Furthermore, partial lesion models (Fitzgerald et al., 2010, Prilloff et al., 2012) may help to determine if a minimum proportion of surviving RGCs and central connections are needed to provide a substrate for LI-rTMS to promote survival and beneficial reorganisation of spared connections.

A further consideration is that the retinofugal pathway lacks the complex excitatory and inhibitory circuitry of the cerebral cortex upon which rTMS is thought to act (Funke and Benali, 2011, Vlachos et al., 2012, Lenz et al., 2014) However, we stimulated the retina, which possesses complex regulatory inhibitory and excitatory circuits that have been compared to those in the cortex (Seung and Sümbül, 2014) and it may therefore provide an appropriate substrate for rTMS if the relevant protocols and models are established. In addition to exploring the role of magnetic field intensity, it will be therefore be important to examine the effect of stimulation frequencies and number of pulses on cell survival and regeneration due to possible
frequency and dose-dependent effects of rTMS on complex circuitry (Volz et al., 2013, Aydin Abidin et al., 2008, Trippe et al., 2009).

Non-invasive brain stimulation techniques for treating neurotrauma

Our result that chronic LI-rTMS does not increase cell survival at 2 weeks is similar to previous reports of other types of non-invasive brain stimulation interventions following complete optic nerve crush injury. For example, tACS stimulation failed to increase RGC survival in the acute phase (1 week post injury) but RGC survival was improved compared to controls at 4 weeks post crush (Henrich-Noack et al., 2013a). The authors suggest tACS may act upon the delayed mechanisms of retrograde cell death. By contrast, another study found that transcorneal electrical stimulation (TES) resulted in increased RGC survival at 1 week post optic nerve crush but this was not sustained at 2 weeks (Henrich-Noack et al., 2013b). These results suggest that different non-invasive brain stimulation methods may have diverse mechanisms of action that are effective at different times in the cell death cascade (early vs. late phase). In one study, rTMS was delivered in the acute post stroke phase (1 hour post stroke) and found decreased apoptosis and improved function. However, stimulation within 24 hours of an injury may be inappropriate, due to abnormal excitability observed in the acute stages post trauma (Griesemer and Mautes, 2007, Johnstone et al., 2013). Yoon and colleagues examined the use of rTMS in the sub-acute stage (4 days post stroke) and found an increase in anti-apoptotic proteins with improved behavioural function (Yoon et al., 2011). Therefore, although our results suggest that LI-rTMS is not neuroprotective in the acute phase post injury, the impact of LI-rTMS on later stages of retrograde cell death should be explored, perhaps in combination with other types of electrical stimulation applied acutely following injury (e.g. TES).
Conclusion
As the mechanisms of action of non-invasive brain stimulation techniques become increasingly well understood through human and animal studies, it is important to continue to explore the potential for previously unconsidered therapeutic effects. The disappointing outcomes for neuroprotection and regeneration following LI-rTMS relative to other non-invasive brain stimulation protocols suggest that LI-rTMS is not adapted to this purpose. Rather LI-rTMS may better be applied to aid neural rehabilitation by modulating the plasticity of spared tissue after injury (Hou et al., 2014). Furthermore, our current and previous work suggests that LI-rTMS may still have a role in the protection and conservation of intact RGCs after trauma, as long as these neurons retain the ability to retrogradely transport BDNF after injury. In addition to exploring the role of magnetic field intensity, it will be important to examine the effect of stimulation frequencies and number of pulses on cell survival and regeneration due to possible frequency and dose-dependent effects of rTMS (Volz et al., 2013, Aydin Abidin et al., 2008, Trippe et al., 2009). In summary, the results from this study help define the therapeutic utility and scope of LI-rTMS treatment and suggest that although LI-rTMS can induce plasticity in intact tissue, it does not induce neuroprotection immediately after severe neurotrauma. Future studies should examine the application of LI-rTMS in more acute and sub-acute stages following neurotrauma and in less severe injury models such as partial lesions.
Chapter 3: Construction and Evaluation of Rodent-Specific rTMS Coils

The results of chapter 2 showed that 12mT LI-rTMS did not induce neural plasticity following neurotrauma. We proposed that 12mT may have been “too low” in the range of LI-rTMS to induce neural plasticity as this is two orders of magnitude lower than HI-rTMS. Therefore to discount the possibility of LI-rTMS failing to induce plasticity due to intensity in our future studies, we aimed to construct and characterise rodent-specific rTMS coils capable of inducing LI-rTMS at one order of magnitude lower than HI-rTMS which retained the spatial resolution of the 12mT rodent-specific coils.


**Introduction**

Transcranial magnetic stimulation (TMS) has excellent potential for modulating human brain plasticity; however, the cellular and molecular mechanisms underlying TMS-induced plasticity remain poorly understood. Rodent models of TMS play a significant role in understanding TMS-induced plasticity mechanisms as they offer a more direct measure of TMS-induced synaptic and non-synaptic plasticity (Tang et al., 2015c). However, one of the main limitations to rodent models of TMS is the lack of rodent-specific TMS stimulator coils. For example, most rodent studies use commercial human coils that are larger than the rodent brain, such as “small” figure of eight (Hoppenrath and Funke, 2013, Vahabzadeh-Hagh et al., 2011) or round coils (Gersner et al., 2011). Whilst the use of such coils allows for stimulation at high intensities used in humans (1-2T), they lack the equivalent spatial resolution (Weissman et al., 1992) (Figure 3.1A). Offsetting coil position can achieve greater stimulation focality (Rotenberg et al., 2010, Vahabzadeh-Hagh et al., 2011), however an alternative approach for rodent TMS is to scale-down coil size to improve focality. Whilst recent work has shown that coil size can be dramatically reduced and maintain high intensity...
capabilities, it still results in relatively unfocal stimulation (Parthoens et al., 2016). In contrast, compromising stimulation intensity for greater focality, rodent-specific coils (circular, 8mm outer diameter) (~12mT) (Figure 1B) have recently been shown to induce structural and molecular plasticity in midbrain and cortical brain regions of mice (Makowiecki et al., 2014, Rodger et al., 2012). However the effects induced by low intensity TMS may not be representative of the changes produced by high intensity stimulation used in human TMS studies (Grehl et al., 2015).

Thus, there is further need to develop a small animal coil that can deliver TMS at higher intensities, whilst maintaining a good degree of spatial resolution (i.e. focality). However, maintaining high stimulation intensities in small coils has physical constraints such as increased thermal and mechanical stress (Cohen and Cuffin, 1991). The stimulation intensities that can be reliably delivered in an experimental setting by rodent-specific coils have yet to be explored. Here we describe two novel rodent-specific TMS coils that deliver stimulation at modest intensities (~100mT) to the rodent brain whilst maintaining the spatial resolution of previous low-intensity rodent coils. These small coils provide an alternative approach to the use of non-focal high intensity human coils or focal low-intensity rodent coils, to investigate TMS neuromodulation in rodents.

**Methods**

*Coil and stimulation parameters*

Two custom circular coils of the same dimensions (8mm height x 8mm outer diameter) with either an air or iron core were constructed (Figure 3.1C). Insulated copper wire (0.125mm diameter, Brocott UK, Yorkshire, United Kingdom) was wound (780 turns) around a steel or plastic bobbin (Inner diameter 4mm, outer diameter 8mm). Coils were wound with a fine wire coil-winding machine (Shining Sun SW-202B, Taipei, China).

Stimulation parameters were controlled by a waveform generator (Agilent Technologies 335141B, California, USA) connected to a bipolar voltage programmable power supply (KEPCO BOP 100-4M, TMG test equipment, Melbourne, Australia).

Current in the coil flowed in a direction that induces an anterior to posterior current across the left rat motor cortex (figure 3.3B) (i.e. posterior to anterior in the coil).
Experiments were conducted at 100% of the maximum power supply output (100V) using custom biphasic waveforms (400µs rise, 400µs fall and 100µs rise) (Agilent Benchlink Waveform Builder, California, USA).

**Magnetic field decay and measurements**

We used a Hall Effect probe to measure the magnetic field magnitude generated by the coils. Coils were fixed to a stereotaxic frame and manipulated around the Hall Effect probe (Honeywell SS94A2D, New Jersey, USA). Measurements of single pulse stimulation were taken in the perpendicular (xy) and parallel (z) axes relative to the main axis of the coil. Due to the axial symmetry of the circular coil, measurements in the x axis also represent the y axis and are therefore referred to as xy. Coil centres were positioned directly above the Hall Effect probe (xy, z=0 mm) and repositioned independently at 1mm increments to a maximum distance of 10mm in each axis (xy_{max}=+10mm, z_{max}=+10mm). The peak Hall Effect voltage from the rising phase of the biphasic pulse was recorded for 4 pulses at each coordinate and averaged to obtain mean field strength as a function of position. Hall Effect voltages were recorded and analysed with data acquisition software (Labchart 6, ADI instruments, Sydney, Australia).

Here we define magnetic field focality as the distance at which the magnetic field is reduced to 50% of the peak.

**Field strength during 1 and 10 Hz stimulation**

Magnetic field measurements (with the Hall Effect probe) were averaged across the first and last 10 pulses of a 600-pulse train delivered at 1 and 10Hz, and stability defined as the ratio of these averages expressed as a percentage. Stability % = (Mean magnetic field of the last 10 pulses/ Mean magnetic field of the first 10 pulses) x 100.

**Temperature measurements**

Coils were fixed to a K type thermocouple (-40° to 260°C, Dick Smith Electronics Q1437, Perth, Australia) and temperature recordings taken every 50 pulses during the 1 and 10Hz protocols.

**Sound measurements**

Attempts were made to measure the sound intensity/sound pressure level (SPL) of the brief clicks emitted by the coils undergoing 1 and 10Hz rTMS using a ½” condensor
microphone (Bruel and Kjaer Type 4134, Sydney, Australia) placed as close as possible to the coil. The microphone was calibrated using a Bruel and Kjaer Type 4231 calibrator. The output of the ½” microphone was viewed directly on an oscilloscope screen (Rigol DS1052E 50MHz, Measurement Innovation, Perth, Australia). It was found that there was a major artefact in the microphone output that was induced by the magnetic field from the coil and this could not be eliminated by shielding. This induced artefact was critically dependent on the spatial relationship between the TMS coil and the recording microphone, with the smallest artefact being present when these were at right angles to each other. Under these circumstances, the signal from the microphone (presumably a mixture of induced artefact and real acoustic signal) had a peak amplitude that corresponded to approximately 75dB SPL (re 20µPa). Because there was no way of separating artefact and acoustic signal in this method, this was thought to be an overestimate of the real sound pressure of the acoustic clicks emitted by the coil. A bioassay method was then used, using two normal hearing human listeners. The sound from the coil inserted into the external ear canal of one ear was matched in apparent loudness to a brief click presented to the other ear using calibrated custom sound generating equipment described in detail elsewhere (Mulders et al., 2011). The duration and spectral content of the clicks were adjusted to match as closely as possible the clicks emitted by the TMS coil.

**Finite element modelling**

Finite element modelling was performed on a high throughput computer cluster consisting of 423 nodes and 4,296 processors using the commercially available Multiphysics 5.0 AC/DC module (COMSOL, Burlington, USA) to give a general estimate of the induced electric field strength within the animal’s brain tissue during the magnetic stimulation. The geometry of the model was based on the coil used empirically, specifically a multi-turn circular copper wire coil (780 turns, inner diameter of 4 mm, wire diameter of 0.125 mm) containing a soft iron (with losses) core. Only the iron core coil was modelled, as this produced the greatest magnetic field and had better magnetic field stability during rTMS (see results below). Simulations were performed by driving the coil with a 100 V input (dI/dT=1.83mA/µs) in the frequency...
domain, using the rise time frequency of the biphasic pulse (2.5 kHz). This method is similar to that used in studies modelling magnetic stimulation of neural tissue, their methods section reviews the equations used for magnetic and electric fields in COMSOL (Bonmassar et al., 2012, Gasca, 2013). Modelling of the electric field was performed using a simplified geometry (Gasca, 2013) of the rat brain, an ellipsoid of 21 x 15.5 x 10.75 mm, taken from a rat brain atlas (Paxinos and Watson, 1982). The skull was modelled with a thickness of 0.7 mm, the average depth of the rat skull (Levchakov et al., 2006). The simulation was performed with the coil positioned 0.25 mm above the centre of the skull. Dielectric properties for human grey matter and bone were used, and taken from the Foundation for Research on Information Technologies in Society dielectric tissue properties database (Hasgall et al., 2014). These dielectric properties have been used in previous studies of magnetic stimulation in rodents (Crowther et al., 2014, Gasca, 2013, Nowak et al., 2011), and are shown in Table 3.1. Incorporating the frequency dependence of tissue is an important consideration, as low frequency properties are controlled by the conduction of electrolytes in extracellular space, while high frequencies initiate several biophysical processes which change the dielectric properties of the tissue (Foster, 2000). Modelling was completed with the magnetic fields (mf) physics interface, and consisted of five domains: the brain, skull, the surrounding air, and a multi-turn coil domain inclusive of the iron core and copper wire. The surrounding air domain was created using a condition that approximates the domain as set to infinity so that boundary conditions do not affect the solution. Geometry was discretized to the “extra fine” mesh setting with a swept mesh for the infinite air domain and a boundary condition mesh set around the iron core.

To compare our iron-core rodent coil with a commercial coil, we ran an additional FEM model on the Magventure MC-B65 butterfly coil placed 7mm above the ellipsoid rat brain model (as described above). The butterfly coil was modelled similarly to other papers but with parameters specific to the Magventure coil, as two sets of five concentric wires with diameters from 35 mm to 75 mm, spaced 5 mm apart, and placed 7 mm from the skull (due to the plastic casing) (Thielscher and Kammer, 2004, Salvador et al., 2015). The coil input was set at 70% of the maximum stimulator output (MSO) (dI/dT of 112 A/µs).
Table 3.1: Dielectric properties used in modelling

<table>
<thead>
<tr>
<th>Material</th>
<th>relative permittivity</th>
<th>relative permeability</th>
<th>conductivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Soft Iron (with losses)</td>
<td>1</td>
<td>4.0 x 10^3</td>
<td>1.12 x 10^7</td>
</tr>
<tr>
<td>Copper</td>
<td>1</td>
<td>1</td>
<td>5.998 x 10^7</td>
</tr>
<tr>
<td>Brain tissue</td>
<td>6.10 x 10^4</td>
<td>1</td>
<td>1.06 x 10^1</td>
</tr>
<tr>
<td>Skull</td>
<td>1.53 x 10^5</td>
<td>1</td>
<td>2.03 x 10^2</td>
</tr>
</tbody>
</table>

Anaesthesia and Electromyography

To determine whether the modest intensities of the rodent-specific coils are suitable for neuromodulation, we delivered sham stimulation (rodent-coil disconnected from power supply) or 10Hz rTMS to the primary motor cortex of Sprague-Dawley rats (n=4, 250-400g males) with the iron-core coil. The iron-core coil was selected as it produced the greatest intensity and reliable stimulation at higher frequencies (see results below). Animals underwent two MEP recording sessions (a sham stimulation session and an rTMS session over 2 consecutive days). Animals were pseudo-randomised into MEP sessions, such that an equal number of animals (n=2) received sham and rTMS in session 1 and 2. Changes in cortical excitability (MEPs) were characterized with single pulse TMS and EMG recordings of the rat forelimb as described by Rotenberg et al (Rotenberg et al., 2010). Briefly, rats were deeply anaesthetised (verified by absence of pinch reflex) with an intraperitoneal injection of ketamine-xylazine (50mg/Kg and 10mg/Kg respectively, Troy Ilium, Sydney, Australia) and placed into an electrically grounded stereotaxic frame. The torso and all points of contact (ear bars and nose bar)
between the animal and the metal frame were insulated with a thin layer of paraffin film to prevent any electrical conductance between the animal and the stereotaxic frame.

Subdermal needle electrodes (13mm 27G, Neuro Source medical, Ontario, Canada) were inserted into the right brachioradialis muscle (recording electrode) and between the 3rd and 4th digit of the right forepaw (reference electrode). Animals were electrically grounded with a single needle electrode inserted into the base of the tail. EMG signals were amplified (x1000), band pass filtered (0.1-1000Hz) (World Precision Instruments DAM50 Bio-amplifier, Coherent scientific, Adelaide, Australia) and acquired at a sampling rate of 40kHz (Powerlab 4/30 ADI Instruments, Sydney, Australia) with Scope software 4.1.1 (ADI instruments, Sydney, Australia). EMG recordings were stored for post-hoc analysis (MEP peak-peak amplitudes). Automated calculation of MEP amplitudes were calculated in a 10 to 20 ms window post single pulse TMS (i.e. MEPs had a latency of 10-20ms post stimulus).

**Single Pulse TMS and rTMS**

A MagPro R30 stimulator equipped with a Magventure BC-65 butterfly coil (Magventure, Farum, Denmark) was used to deliver single pulse TMS over the left motor cortex. MEP recordings were rapidly generated at 75% of machine stimulator output immediately before and after sham or rTMS stimulation (an intensity known to produce suprathreshold stimulation in rats anaesthetised with ketamine-xylazine (Vahabzadeh-Hagh et al., 2011). Single pulse parameters consisted of 8 pulses with an inter-stimulus interval of 7 seconds. Immediately following baseline MEP recordings, the iron-core rodent coil (base wrapped in a thin layer of paraffin to insulate the iron-core from the animal) replaced the figure of 8 coil and was placed on the rat head (lightly touching the skull), such that the coil windings overlaid the left motor cortex. This coil position was chosen as the greatest induced current occurs under the windings and not at the coil centre. Stimulation consisted of 3 minutes of sham or 10Hz rTMS (total of 1800 pulses). Immediately after stimulation, MEPs were recorded (as described above). We chose to record MEPs immediately following stimulation as studies in humans suggest that effects are maximal within the first 20-30 minutes following stimulation. In addition, we wished to avoid continuous dosing of
anaesthetic, which results in fluctuations of cortical excitability that is different for each animal. Therefore to maximise the consistency of MEP measurements between animals, we restricted our MEP measurements to within a 30 minute window where cortical excitability and anaesthesia depth were stable after a single anaesthetic injection (as confirmed in sham animals). At the end of each MEP recording session, anaesthesia was reversed with an intraperitoneal injection of atipamazole (1mg/Kg, Troy Ilium, Sydney Australia) to increase the survival of the animals. All Experimental procedures were approved by the UWA Animal Ethics Committee (03/100/1371).

**Data analysis**

Statistical analysis was performed with SPSS® (IBM, New York, USA). All means are presented with their respective standard error of the mean.

For magnetic field stability, a multivariate ANOVA was conducted to detect coil type (dependent variable) differences in magnetic field stability at 1 and 10Hz (independent variables). For MEP amplitudes, a ratio of the mean post stimulation MEP amplitude relative to the mean sham MEP amplitude was calculated and log transformed for analysis. A paired t-test was conducted to detect whether rTMS (dependent variable) altered MEP amplitudes (independent variable). We also used 95% confidence intervals to support our use of parametric analysis.
Figure 3.1. A schematic diagram of a commercial coil positioned to stimulate the left motor cortex (B). A schematic of a commercial 70mm round coil placed over a rat head, which encapsulates the entire brain (A). Birdseye (top) and side on views (bottom) of the novel air-core (left) and iron core (right) 8mm round coils (C). A diagram of the custom biphasic waveform input from the waveform generator (top) and resulting output as measured by a Hall-effect sensor (bottom) (D).

Results

Magnetic field strength –peak values and decay
Magnetic field strength in the xy and z axes is illustrated in Figure 3.2. The iron-core coil produced a greater peak magnetic field (119.05mT±0.42) relative to the air-core coil (89.50mT±6.56) but with decreased focality. Half-maximum field occurred at ~1.2mm\textsubscript{z} axis, ~3.5mm\textsubscript{xy} axis (air-core) and ~2mm\textsubscript{z} axis, ~4mm\textsubscript{xy} axis (iron-core).

Changes in coil temperature
Temperature measurements over 600 pulses of 1 and 10Hz stimulation showed frequency and coil type dependent changes (Figure 3.2C). Increases in coil temperature for 1Hz stimulation peaked at 5.8 °C ±0.40 (Air-Core) and 1.67 °C ±0.38 (Iron-
Peak increases in coil temperature for 10Hz stimulation were 17.43°C±1.07 (Air-Core) and 3.57°C±0.47 (Iron-Core).

Similarly, the time for coil temperatures to return to baseline after 600 pulses of 1 and 10Hz stimulation showed frequency and coil type dependent changes. Times to return to baseline (min:secs) for 1Hz were 7:42 ±0:06 (Air-Core) and 2:49 ± 0:03 (Iron-Core) and 9:39 ±0.04 and 5:53 ±0:02 (Iron-Core) for 10Hz.

Change in temperature of the iron-core coil undergoing 1800 pulses of 10Hz stimulation for neuromodulation and EMG assessment (see below) peaked at 6.8 °C ±0.24.

**Sound emission from coils**

Measurement of the sound pressure level at the base of the coils undergoing rTMS with a sound level meter sound failed to give an accurate measurement due to the biphasic stimulus artefact induced in the microphone by the rTMS. Using the bio-assay method, an approximation of the peak sound intensity of the TMS clicks emitted by the coils was ~26dB SPL.

**Magnetic field stability**

A MANOVA on the magnetic field stability measurements (Figure 3.2D) showed statistically significant coil differences at 10Hz stimulation (p<0.01) but not at 1Hz stimulation (p=0.084). At 1Hz stimulation, both coils showed high stability (100.03%±1.03 (Air-Core) and 99.70%±0.93 (Iron-Core)) at the end, relative to the beginning, of the stimulation train. However, at 10Hz stimulation, magnetic field stability was reduced (89.20%±1.05 as a result of the reduction in magnetic field intensity towards the end of stimulation) in the air-core coil whereas there was no change in stability for the iron-core coil (99.65%±1.02).
Figure 3.2. Characterisation of coil properties. Magnetic field decay in the z (A) and xy (B) axes where 0 is the centre of the coils, shows the iron-core coil produced a greater peak magnetic field (119.05mT) than the air-core coil (89.50mT) with a trade-off of focality. Half-maximum field occurred at ~1.2mm, ~3.5mm (air-core) and ~2mm (iron-core). Changes in the iron-core coil temperature during 600 pulses of 1 and 10Hz rTMS (C) shows tolerable changes in temperature (≤Δ5˚C) at both frequencies. 10 Hz stimulation with the air-core coil resulted in a large temperature change (~Δ17.5˚C). Magnetic field stability (D) shows the iron-core coil shows high stability at both 1 and 10Hz stimulation. Magnetic field stability for the air-core coil at 10Hz significantly decreased (p<0.001) at 10Hz.

**Finite element modelling**

Results from the FEM simulation found a magnetic field strength of 115mT directly below the windings of the coil. The magnetic field distribution (mT) in the xy (coronal) plane is shown in Figure 3A, and the current density is represented by the arrows in Figure 3B. The maximum electric fields simulated within the skull and brains were 85 V/m and 12.7 V/m, respectively (Figure 3 C&D). These were located below the windings of the coil, similar to the placement of the coil used for the MEP recordings.
The estimated electric field was > 10 V/m up to a depth of 0.7 mm, > 5 V/m to 1.4 mm, and > 1 V/m to 3.3 mm.

Figure 3.3. Finite element modelling of the iron-core coil. The magnitude of the magnetic field (mT) and magnetic flux density in the xy plane (A). The arrows represent the direction of the current density separated in 15 bins. The induced current density with the brain, shown by normalized arrows separated into 12 equal bins for the xy grid and 4 in the z direction (B). Electric field magnitude (V/m) in a coronal slice of the ellipsoids representing the skull and brain below the coil windings (C). The inset shows an enlarged view of the electric field at the brain and skull interface. The simulated electric field strength within the skull and brain as a function of depth (D). The inset shows electric field strength with the brain domain on a different y-axis scale.

The peak electric fields in the rodent model under the Magventure coil were 1 order of magnitude larger than our rodent coils at 856 V/m in the skull and 224 V/m in the brain (Figure 3.4A). The electric field induced was also larger with an estimated electric field of >150V/m at a depth of 10mm from the surface (Figure 3.4B).
Figure 3.4. Finite element modelling of the Magventure BC-65HO butterfly coil. The induced electric field (V/m) in a coronal slice of the ellipsoid model (A). The simulated electric field strength within the skull and brain as a function of depth (B).

**10Hz rTMS and cortical excitability**

Following sham stimulation, mean MEP amplitude was 98.25% ± 3.207 of the mean baseline MEP amplitude. Following 10Hz rTMS with the iron-core coil, MEP amplitude was 157.1% ± 15.92 of the mean baseline MEP amplitude. A two-tailed paired t-test was conducted on the log10 transformed ratios (post stimulation amplitude/baseline amplitude) (Figure 3.5) revealed a significant difference between sham and rTMS (mean=0.198, SD=0.116) conditions; t=3.403, df=3, p=0.042.
Figure 3.5. Characterisation of MEP’s before and after 10Hz rTMS to the anaesthetised rat motor cortex with the iron-core coil. Raw EMG traces of sham (top) and active (bottom) rTMS (A). Log₁₀ transformation of MEP ratios (post stimulation/baseline) recorded in the right forepaw after 3 minutes of Sham or 10Hz rTMS to the left motor cortex. rTMS significantly increased MEP ratios relative to sham stimulation (p<0.05) (B).

Discussion

We have developed and characterised two novel rodent-specific TMS coils that can deliver greater stimulation intensities than previous rodent-specific coils similar in size (~12mT) (Rodger et al., 2012, Makowiecki et al., 2014). As expected, the addition of an iron core increased field strength relative to the air core coil (Epstein and Davey, 2002) but with a trade-off between greater magnetic field penetration and decreased focality of the iron-core relative to the air-core coil (Deng et al., 2013). Finite element modelling of the iron-core coil undergoing rTMS suggests the induced electric field induced in a simplified rat brain is approximately 1 order of magnitude lower than commercially available human stimulators. Unlike sham stimulation, 10Hz rTMS with the iron-core coil significantly increased MEP amplitudes relative to baseline.
Our results show that the iron-core coil displays good temperature and magnetic field stability at both 1 and 10Hz stimulation, whereas, the air-core showed a large increase in temperature and decrease in magnetic field stability at 10Hz. We attribute the corresponding reduction in field strength to a temperature-related increase in resistance within the copper coil wire. Greater temperature and field stability in the iron-core coils suggest that the core potentially acted as a heat-sink, minimising heat retention in the copper coil windings. By contrast, temperature increased in the air-core coil most likely because air is a poor conductor of heat. However, it is important that any additional rTMS stimulation protocols be evaluated prior to use, as the efficacy of the iron-core as a heat sink is likely to diminish with higher frequencies (e.g. theta burst protocols), repeated blocks of stimulation or longer durations which may cause excessive heating in the coil with potential harm to the rodents.

Given the greater magnetic field output and thermal stress performance of the iron-core coil, we suggest the iron-core coil is more suitable for use in rodent studies, particularly at high frequency stimulation.

Decreasing coil size has raised the question of stimulation efficiency as smaller coils induce proportionally smaller electric fields. Our calculations are consistent with a model of a commercial TMS stimulator and coil over a mouse brain which found a peak magnetic and electric field of 1.7 T and 132 V/m respectively, approximately 1 order of magnitude larger than our small custom coils (Crowther et al., 2014). Furthermore, our calculations suggest the induced electric field from the iron-core coil results in approximately 10% of the electric field needed for axonal suprathreshold stimulation (100V/m). Therefore to investigate whether the modest magnetic field/electric field strength delivered by the iron-core coil (~120mT) could induce neuromodulatory effects, we delivered 10Hz rTMS to a small number of anaesthetised rats combined with EMG recordings to quantify possible changes in MEPs. The iron-core coil was selected as it not only produced the strongest field strength but also showed greater temperature stability and stimulation reliability with high frequency rTMS. Our results showed 10Hz rTMS significantly increased MEP amplitudes immediately after stimulation, with a mean increase of approximately 57% relative to baseline recordings. These findings are in line with both human (Arai et al., 2007, Jung et al., 2007) and rodent studies (Hsieh et al., 2014) that showed increased MEP amplitudes...
with subthreshold high frequency rTMS delivered with commercial stimulators and coils. However, although our results provide preliminary evidence that these modest magnetic/electric field intensities can induce neuromodulatory effects in rats, further characterisation of changes in cortical excitability and molecular markers are needed. Unlike HI-rTMS, which involves NMDA and AMPA receptors as elegantly demonstrated by recent publications from the Vlachos and Funke research groups (Labedi et al., 2014, Lenz et al., 2014, Lenz et al., 2016), low and moderate intensity rTMS as delivered here is likely to be subthreshold for action potentials, and therefore involve different mechanisms such as changes in intracellular calcium and BDNF levels (Grehl et al., 2015, Makowiecki et al., 2014). By providing a full characterisation of the biophysical properties of our small coils, our report will enable future studies to examine in more depth the molecular and cellular mechanisms involved in the induction of cortical plasticity. It will also be important to determine whether the plasticity induced by these small coils is unilateral or bilateral, as well as characterize changes in corticospinal excitability with complete input-output curves, time course of changes and frequency-specific effects.

Approximation of the induced electric field focality of the iron-core coil with FEM modelling showed that the induced electric field peaked below the windings of the coil, and is in line with FEM modelling of commercial coils in spherical head models (Deng et al., 2013). Furthermore, the spread of the electric field was highly localised and undergoes a rapid decay to <1V/m within millimetres of the peak field. An estimate of stimulation penetration shows that the induced electric field remains above 1V/m at a distance of 4mm below the surface of the coil. Accounting for skull thickness (0.7mm), this equates to an electric field greater than 1V/m to a depth of ~3.3mm in the rat brain. This is in contrast to the induced electric field produced with a commercial butterfly coil, which resulted in a greater peak electric field (224V/m) and more widespread electric field such that the electric field was >150V/m at a depth of 10mm from the surface of the brain and encapsulated the entire brain. This is similar to the electric field modelling with the commercial Cool-40 Rat coil, which induces a peak electric field of 220V/m with a penetration of ≥50V/m at a depth of ~10mm (Parthoens et al., 2016). These results suggest that although our coils produce weaker electric fields, they induce more focal stimulation. Given the rapid electric field
decay with our coils, it is likely that stimulation is restricted to the cortical and superficial sub-cortical layers of the rat brain (e.g. pyramidal cell layer of the hippocampus) depending on the coil position and orientation. Due to decreased skull thickness and brain size, we expect reduced focality/spatial resolution if used in smaller rodents, such as mice. Whilst this decreases the ability to target specific brain regions, it increases the ability to target deeper brain structures.

A limitation of this study was the need to replace the rodent-specific coil after rTMS with a human figure of 8 coil to induce MEPs. However, due to the subthreshold nature of our rodent-specific coils, eliciting MEPs with a stronger human coil was essential. The use of an unplugged coil to deliver sham is a potential limitation of the study. Whilst the unplugged coil sham maintains the mechanical stimuli of coil placement on the head and background auditory stimuli from the stimulator equipment, it lacks the auditory stimuli of the click sound produced by the coil during active TMS. Approximation of the sound pressure level generated by the air and iron-core coil undergoing 10 Hz rTMS was ~26dB at the base of the coil. Previous rodent studies suggest that at this intensity, the low frequency sound emitted by the coils is below the hearing threshold of mice (Fernandez et al., 2010) and close to the threshold for rats (Borg, 1982). However, as sound intensity decreases with distance (the inverse square law), it is likely that the ~ 26dB at the base of the coil is an overestimation of any sound perceived in the ears of the animal and would be dependent on coil position. Furthermore, it is unlikely that the auditory and small vibration component of active stimulation would induce sensory (e.g. shifts in attention and alertness) and/or placebo (e.g. the belief that one is receiving active stimulation) side effects (Duecker and Sack, 2015), in animals (particularly anaesthetised animals as in this study).

FEM simulations using simplified spherical models are useful when approximating the general electric field properties in neural tissue. However, simplified models come with limitations, which have been addressed in other modelling papers. One of these is that isotropic tissue conductivities are used (Miranda et al., 2013), though a recent paper found no substantial differences in the electric field distribution between models with isotropic versus anisotropic conductivities (Salvador et al., 2015), and another found only weak increases in electric field strength due to the anisotropy of brain tissue.
(Opitz et al., 2011). Furthermore the electric field estimations (which neglect local maxima at the gyral folds) do not take the radial electric field component into account, and are altered (and likely improved) in more detailed models (Salvador and Miranda, 2009, Thielscher et al., 2011). Whilst the rat and mouse cortex lacks folding and is relatively smooth, estimations of the electric field should be interpreted with care when extrapolating to regions like the cerebellum (where folding does occur in rats and mice) or in the brains of larger rodents such as guinea pigs which have more complex cortices.

In conclusion, we provide an alternative method to deliver TMS to rodents by constructing small rodent-specific TMS coils capable of delivering modest stimulation intensity whilst maintaining stimulation focality. Our results show different field strengths, penetration, focality and performance for each coil that need to be considered prior to coil selection. Whilst our coils induce modest magnetic and electric fields, we have shown such field strengths can induce neuromodulatory effects. Therefore, we suggest these moderate intensity rTMS coils provide a useful tool for the preclinical investigation of TMS plasticity in rodents.
Chapter 4: Low-intensity repetitive magnetic stimulation modulates skilled motor learning in mice.

To further investigate whether LI-rTMS modulates neural plasticity, we examined whether LI-rTMS would modulate behaviour in the healthy CNS, similar to modulation of motor behaviour with HI-rTMS in humans. Using the coils developed in chapter 3, we paired 120mT priming or consolidation LI-rTMS with skilled motor learning in adult mice. We used western blot and ELISA molecular biology at the end of the behavioural experiments to determine the potential cellular mechanisms underlying any behavioural change.

Introduction

Repetitive transcranial magnetic stimulation (rTMS), a non-invasive form of brain stimulation is widely used to modify neural plasticity in clinical and non-clinical populations. Intermittent theta burst stimulation (iTBS), a commonly used rTMS protocol, has gained considerable interest as it can induce long term potentiation (LTP) like plasticity in the human motor cortex (Huang et al., 2005), with cortical excitability changes persisting up to 60 minutes after a single stimulation (Wischnewski and Schutter, 2015).

In addition to modulating cortical excitability, iTBS can be used to modify motor learning (Teo et al., 2011, Stöckel et al., 2015, Läppchen et al., 2015). This can be achieved by delivering rTMS as a priming stimulus (i.e. rTMS given prior to motor training) (Teo et al., 2011, Läppchen et al., 2015) or as a consolidating stimulus (i.e. rTMS given after motor training) (Stöckel et al., 2015). However the potential mechanisms underlying the interaction of iTBS induced neural plasticity remain unclear.

Rodent models provide a valuable adjunct to human rTMS studies, due to the powerful but invasive techniques available to investigate plasticity and its associated mechanisms (Tang et al., 2015c). Similar to humans, iTBS has been shown to induce neural plasticity in the motor cortex in rodents using several techniques including increased motor evoked potential amplitudes, changes in sensory-motor learning (Mix
et al., 2010) and in immediate early gene expression (Zif68) (Aydin Abidin et al., 2008). Potential mechanisms of iTBS induced plasticity in rodents come from histological and molecular studies, which suggest alterations in both excitatory and inhibitory activity (Labedi et al., 2014, Hoppenrath and Funke, 2013, Trippe et al., 2009) and changes in brain derived neurotrophic factor (BDNF) expression (Castillo-Padilla and Funke, 2015). However, whether low-intensity rTMS can modulate motor learning and whether similar cellular mechanisms are involved are unknown. Here we investigated whether chronic iTBS delivered at a low intensity (120mT) to the motor cortex immediately prior to or after daily pellet reaching training would prime or consolidate motor learning in adult mice over 10 consecutive days. Furthermore, potential molecular and biochemical mechanisms underlying priming iTBS induced plasticity at the conclusion of 10 days motor learning were investigated with molecular biological techniques to assess changes to excitatory (Glur1, Glur2, PSD95), inhibitory (Gephyrin) and general synaptic plasticity (ARC and BDNF) proteins.

The selected synaptic proteins were chosen as they have potential overlap between synaptic plasticity and rTMS-induced plasticity. Given the key involvement of AMPA receptors in HI-rTMS-induced plasticity (Lenz et al., 2014, Vlachos et al., 2012), we selected protein targets indicative of AMPA receptor activity. PSD95 is a key scaffolding protein in the excitatory post-synaptic compartment and regulates the retention of post-synaptic AMPA receptors (Keith and El-Husseini, 2008). Altered PSD95 expression is linked to a change in the excitation/inhibition balance at the synapse. Growth associated protein 43 (GAP43) is also present in this compartment, and is essential in regulating AMPA receptor density at the postsynaptic membrane (Han et al., 2013). Our final markers of AMPA receptors are the AMPA receptor subunits Glur1 and Glur2, whose differential expression is linked to synaptic plasticity (Chater and Goda, 2014). As an additional marker of synaptic plasticity, we used the activity-regulated-cytoskeletal protein Arc as it is involved in both LTP (Guzowski et al., 2000, Messaoudi et al., 2007), LTD (Park et al., 2008, Waung et al., 2008) and metaplasitcity (Shepherd et al., 2006, Béique et al., 2011) and is believed to be regulated by excitatory activity (Kawashima et al., 2009). BDNF was selected given its upregulation with LI-rTMS and HI-rTMS (reviewed in chapter 1).
As a marker of plasticity at the inhibitory synapse, we investigated gephyrin, a scaffolding protein at the post-synaptic membrane, and is the protein to which GABA\textsubscript{A} receptors anchor (Kneussel and Betz, 2000, Tyagarajan and Fritschy, 2014). Moreover, gephyrin expression is modulated with HI-rTMS (Lenz et al., 2016). Combining behavioural testing with molecular biology will allow us to determine the effect of chronic LI-rTMS (10 consecutive days) at the systems level (skilled motor learning) and the potential cellular mechanisms (synaptic proteins) underlying LI-rTMS-induced plasticity.

**Methods**

**Animals**

10 week old male C57Bl/6J mice (*Mus Musculus*) (n=48, UTAS colony originally derived from Jackson laboratory stocks) were group housed on a 12-hour light/dark cycle. Two days prior to commencement, animals were food restricted to 90% of their baseline weights (~2 hours of food per day given after behavioural testing) and had water *ad libitum*. 24 hours after the end of experimentation, mice were euthanased with an overdose of pentobarbitone (>160mg/kg i.p injection). All procedures were approved by the Animal Ethics Committee of the University of Tasmania.

**Custom rodent rTMS coil**

A custom rodent-specific coil was used to deliver more focal rTMS and avoid direct stimulation of other cortical regions involved in motor function (e.g. cerebellum) that may have occurred with larger commercial coils (characterised in chapter 2; Tang et al., 2016). Briefly, insulated copper wire (0.125mm diameter, Brocott UK, United Kingdom) was wound (780 turns) around a steel bobbin (inner diameter 4mm; outer diameter 8mm). Coils were wound with a fine wire coil-winding machine (Shining Sun SW-202B, China).

Monophasic stimulation pulses (400\(\mu\)s rise time) were delivered by a waveform generator (Agilent 33514B, USA) connected to a bipolar voltage programmable power supply (KEPCO BOP 100-4M, USA). Experiments were conducted at 100% of the maximum power supply output (±100V) (Agilent Benchlink Waveform Builder, USA). Peak magnetic field strength was 120mT at the surface of the coil (measured with a
Hall-effect probe Honeywell SS94A2D, USA). Maximum stimulation equated to a dB/dT of 400T/s.

**Repetitive Transcranial Magnetic Stimulation**

rTMS or sham stimulation (handling control) was delivered daily, for 10 consecutive days to the motor cortex contralateral to the dominant/trained forelimb of lightly restrained awake mice, by placing the mouse in a tapered plastic film restraint bag with a breathing hole at one end (Able Scientific, Australia). The TMS coil was positioned over the mouse cranium, such that the coil windings overlaid the dominant motor cortex, with the coil offset laterally to avoid direct stimulation of the non-dominant motor cortex (figure 1). During stimulation, the coil was held ~1mm over the mouse cranium. 600 pulses of iTBS (3 pulses of 50Hz delivered for 2s, repeated at 5Hz) (Huang et al 2005), lasting 190 seconds was delivered once daily for 10 consecutive days, either immediately before in the priming group (n=16 per group) or after motor training in the consolidation group (n=8 per group). Sham stimulation consisted of manual restraint for 190s as described above and placement of an unplugged coil over the cranium.

**Skilled Motor Training: Single-Pellet Reaching Task**

The single pellet-reaching task is a skilled forelimb behavioural task that is analogous to throwing darts or shooting basketballs in humans (Chen et al., 2014). Briefly, a single animal was placed into a clear chamber (20cm tall, 15cm deep and 8cm wide). A vertical slit (10mm wide) in the centre of the chamber allowed the animals to retrieve a food reward (2.5mm diameter, 20mg chocolate flavour grain pellet, Bio-Serv, USA) placed on a food tray in front of the slit. Pellets were replaced by the experimenter immediately after (i) the pellet was grasped by the animal or (ii) the pellet was displaced due to the animals reaching attempt.

Animals underwent pre-training (“shaping”) once a day until a baseline level (30 reaches made within a 20 minute training session) was achieved. In addition, the animals’ individual learning abilities were defined based on the number of shaping days needed until baseline level was achieved (“fast learner”= 1 day of shaping required, “intermediate learner”= 2 to 3 shaping days required or “slow learner”=4
shaping days required). Animals that did not reach baseline level after 4 days shaping were excluded from further testing. For shaping, pellets were placed in the centre of the slit, allowing the animal to reach with both forelimbs. The dominant forelimb was defined as the forelimb used in >70% of reaching attempts. Groups were counterbalanced such that an equal number of learning abilities and paw preferences were placed into sham and rTMS groups.

Motor training consisted of 30 reaches or 20 minutes of training, whichever was achieved first. The pellet was placed to one side of the slit midline such that the animal could only collect the pellet by reaching with its pre-determined dominant forelimb (preventing reaching with non-dominant hand and tongue). Reaching attempts were scored as a success (dominant forelimb grasped and retrieved pellet and was fed into the animals mouth), a drop (dominant forelimb grasped and retrieved pellet but was dropped before feeding into the animals mouth) or a fail (an attempt with the dominant forelimb that missed or misplaced the pellet). Animals received one session of motor training per day for 10 consecutive days.

**Molecular Biology Tissue Preparation**
24 hours after the last period in the testing chamber, mice were terminally anaesthetised with 160mg/kg pentabarbitone sodium. Brains were rapidly removed and dissected for the hippocampus and overlying cortex (a region containing both motor and somatosensory cortex). Samples were taken from the dominant (i.e. stimulated) hemispheres. Samples were snap frozen with liquid nitrogen and stored at -80°C.

**Western Blotting**
Samples were homogenised in 1mL of ice-cold lysis buffer designed to maximise yield of BDNF (Szapacs et al., 2004) (100 mM PIPES pH7, 500 mM NaCl, 0.2% Triton X-100, 2mM EDTA) containing EDTA-free protease inhibitor cocktail (Roche Biochemicals). Lysates were centrifuged (3320 x g at 4°C for 1 hour) then supernatants stored at -80°C. Protein levels were quantitated via the bicinchoninic acid assay as per the manufacturer’s instructions (Thermo Fisher), and equal amounts (approx 10µg) loaded onto 10% or 12% Tris-glycine reducing SDS-PAGE gels. Gels were transferred onto PVDF membrane (Millipore), blocked with PBS+0.1%Tween containing 5% skimmed
milk powder and probed with antibodies as follows: PSD95 (n=4 per group, Abcam Ab2723, 1:7000); GAP43 (n=4 per group, Sigma GAP-7B10, 1:1000); GluR1 (n=4 per group, Neuromab N3551/75-327, 1:1000); GluR2 (n=4 per group, Neuromab L:21/3275-002, 1:1000); Arc (n=4 per group, Abcam Ab2382, 1:2000); Gephyrin (n=10 per group, Millipore Ab5725, 1:5000) and normalised to the expression of Beta-actin (Sigma A2228, 1:50000). Membranes were incubated with peroxidase-conjugated secondary antibodies to rabbit or mouse (Dako) and imaged with chemiluminescent substrate (Millipore 1:20000).

**BDNF ELISA**

Priming tissue lysates (priming iTBS n=8, priming sham n=9) were assayed using a commercial sandwich ELISA kit as per manufacturer’s instructions (Chemikine, Millipore). BDNF levels were normalised to total protein concentration (BCA protein assay).

**Data Analysis and Statistics**

Behavioural data for each training session was analysed for skill accuracy, speed and learning. Accuracy was defined as the percentage of successful reaches relative to the total number of reaches made. Motor learning was defined as the change in accuracy over time. As sham conditions for priming and consolidation were not identical, we chose to compare priming and consolidation against their respective sham groups rather than combine the shams into one group.

We used the statistics program R (version 3.1.1) running nlme package v3.1-120 (Pinheiro et al., 2016) and lme4 package v1.1-7 (Bates et al., 2014) to perform a linear mixed effects analysis of the relationship between pellet reaching accuracy, time and treatment with TMS. Preliminary model fitting used Akaike’s Information Criterion (AIC) and F-tests to compare models. As fixed effects, we entered time, treatment (iTBS), weight loss and shaping speed (fast/intermediate/slow learners) into the initial model, and included a term for interaction between time and treatment. As random effects, we used random intercepts and random slopes for subjects (individual mice).
A similar mixed model analysis was performed on speed; however the dependent variable was log transformed and a quadratic term entered for time (day-squared). As fixed effects, we entered time, time-squared, treatment, weight loss and shaping speed into the model, and included a term for interaction between time and treatment, and a term for interaction between time-squared and treatment. As random effects, we used random intercepts and random slopes for subjects (individual mice).

For molecular analysis, Q-Q plots and Forsythe’s test were used to test the assumptions of data normality and equal variance respectively with SPSS® (version 20, IBM, USA). Where appropriate data were analysed with unpaired t-tests or Mann-Whitney U tests. Data was graphed with Graphpad Prism (Version 6, Graphpad Software, USA).

**Results**

*Skilled reaching motor skill*

In both priming and consolidating groups, time significantly affected accuracy of reaching (p<0.001), with mice becoming slightly more accurate (0.9-1.5%) each day in performing the task over the 10-day training period. Shaping speed (days required to reach baseline training level) did not significantly affect accuracy. Weight loss percentage showed a significant effect (p=0.03) upon skill accuracy, but only to a small extent (<0.5%).

*Priming LI-rTMS*

When LI-rTMS was given before motor training (n=16 per group), there was a significant effect (p=0.02) of stimulation (Figure 4.1 A), with priming LI-rTMS increasing accuracy by 8.5% ± 3.4 compared to sham stimulation. However, priming LI-rTMS did not alter the rate of learning (p=0.24). To confirm the increase in accuracy with LI-rTMS, we applied a simplified statistical model, removing the non-significant term for interaction between stimulation and time. In this simplified model, both stimulation
(p=0.04) and time (p<0.0001) continued independently to show a significant effect upon accuracy.

To further investigate the effects of priming LI-rTMS on accuracy, we examined whether fast (n=3), intermediate (n=7) and slow learners (n=6) were affected differently. There was a greater increase in accuracy with priming LI-rTMS on fast (5.3%) and intermediate learners (6.8%) with a very small effect on slow learners (0.7%), however these effects did not reach statistical significance (p=0.23, p=0.29 and p=0.89 respectively).

Consolidating LI-rTMS

When LI-rTMS was given immediately after the end of each motor training session (n=8 per group), there was no significant effect of stimulation upon accuracy (p=0.25) (Figure 4.1 B) or the rate of learning (stimulation*time) (p=0.12). To confirm that there was no change in skill accuracy with consolidating LI-rTMS we removed the stimulation*time interaction from our statistical model. In this simplified model, stimulation did not affect skill accuracy (p=0.22). In both models we observed the expected strong effect of time upon accuracy (p=0.0002).

Speed of reaching did not increase with either time or treatment

Similar modelling against speed of reaching in both priming and consolidating LI-rTMS groups indicated that time did not significantly affect reaching speed (p=0.06) (mice did not become faster at performing 30 reaches as the testing days progressed), and that stimulation did not significantly affect speed (p=0.87) (Figure 4.1 C and D).
Molecular Analysis of Priming LI-rTMS Cortical Tissue

To explore the long term molecular basis of the observed changes in motor learning with priming LI-rTMS we conducted western blot and ELISA analysis, on cortical tissue collected from the stimulated cortex, 24 hours after the last training session (i.e. collected on day 11).

Western Blots

Analysis of relative protein expression showed no significant difference between sham and LI-rTMS primed cortical tissue for Glur1 (U=5, p=0.486), Glur2 (U=4, p=0.343), ARC (U=8, p=0.999, PSD95 (U=3, p=0.200), GAP43 (U=6, p=0.686) or Gephyrin (t (18)=0.024, p=0.841) (Figure 4.2).
Figure 4.2. Western blot analysis of cortical tissue from animals receiving priming LI-rTMS and motor training. No significant difference in the expression of Glur1 (n=4 per group) (A), Glur2 (n=4 per group) (B), PSD95 (n=4 per group) (C), GAP43 (n=4 per group) (D), ARC (n=4 per group) (E) or Gephyrin (n=10 per group) (F) between sham and priming LI-rTMS groups (p>0.05). Error bars represent SEM.
Figure 4.3. Mean BDNF expression assessed 24 hours after the last priming rTMS and motor training session. No significant differences between priming sham (n=8) and priming LI-rTMS animals (n=9) (p>0.05). Error bars represent SEM.

**BDNF ELISA**

Mean BDNF concentrations (pg/µg of protein) were similar between sham stimulated and priming LI-rTMS animals (0.220 ± 0.032 and 0.278 ± 0.036 respectively) (Figure 4.3). An unpaired t-test confirmed no statistically significant difference between mean sham and priming LI-rTMS BDNF concentrations t(15)=1.121, p=0.243.

**Discussion**

Our findings demonstrate that priming but not consolidating iTBS delivered at a low-intensity (120mT) interacts with skilled motor training. Priming LI-rTMS significantly increased skill accuracy, without altering the rate of learning (increase in accuracy over time), providing a transient improvement in performance. Furthermore, molecular and biochemical analysis of the chronic primed cortex showed no change in the protein expression of key synaptic proteins and BDNF.
Priming LI-rTMS resulted in a significant transient increase in skill accuracy but did not affect the rate of learning over 10 days compared to sham stimulated animals. Our results contrast with previous priming rTMS using iTBS in both animals and humans, in which higher rTMS intensities altered the rate of learning. Rats receiving priming iTBS at a moderate intensity learned a sensorimotor task significantly faster than sham stimulated animals (Mix et al., 2010). Similarly, in humans, high intensity priming iTBS combined with a simple ballistic motor task resulted in an increased rate of improved performance but did not alter their baseline performance (Teo et al., 2011). Taken together the studies suggest that low and high intensity iTBS may target different aspects of motor learning: high intensity iTBS can be used to modify the rate of motor learning but does not improve performance, whereas low intensities can transiently modify task performance without affecting learning.

Interestingly, applying LI-rTMS as a consolidating stimulus did not alter motor performance or motor learning. Previous studies in humans have shown the ability of inhibitory HI-rTMS to degrade motor learning (Baraduc et al., 2004, Muellbacher et al., 2002). In line with our results, iTBS at high intensities during the consolidation phase of motor training does not change motor performance compared to sham stimulation in humans (Agostino et al., 2008, Jelić et al., 2015). Furthermore, the lack of enhancement with consolidating LI-rTMS may not be surprising given the proposed mechanism for HI-rTMS, where rTMS is given prior to motor training to increase cortical excitability to facilitate motor improvement (Reis et al., 2008) (i.e. increased motor performance is a consequence of priming rTMS-induced changes to cortical excitability) (Tang et al., 2016). Therefore the similar absence of neuromodulation to a consolidation stimulus highlights the importance of rTMS intervention timing (regardless of intensity) and suggests that the clinical potential rTMS to enhance motor behaviour is restricted to a priming intervention only.

In humans, four days of iTBS to the dorsal premotor cortex prior to daily mirror training resulted in significantly less improvement compared to controls (Läppchen et al., 2015). The authors suggest that priming iTBS has a metaplastic interaction with subsequent motor learning, such that high synaptic activity increases the threshold for subsequent LTP and decreases the threshold for LTD (Abraham and Bear, 1996). Our results suggest that low intensity priming iTBS has a non-metaplastic interaction with
subsequent motor learning as the motor learning curves were not different between sham and priming rTMS animals. If a metaplastic interaction occurred, we would have observed that priming iTBS had a decreased rate of learning relative to sham or an initial increase in learning followed by a decrease in the rate of learning. Although Lapchen and colleagues stimulated the dorsal premotor cortex rather than the primary motor cortex, another key difference between our studies is stimulation intensity. The use of lower intensities may have prevented a metaplastic interaction as weaker stimulation may be sufficient to facilitate motor improvement without inducing metaplastic mechanisms (e.g. increased LTP induction threshold).

Potential mechanisms underlying rTMS-induced plasticity as revealed by animal and experimental models include both synaptic and non-synaptic mechanisms which are not mutually exclusive (Tang et al., 2015c). Recent experimental models have shown direct evidence of synaptic mechanisms with high frequency magnetic stimulation (Vlachos et al., 2012, Lenz et al., 2014, Lenz et al., 2016). The authors suggest a spike timing-dependant plasticity like mechanism, whereby the arrival of the rTMS–induced action potential of the pre-synaptic neuron coincides with the rTMS-induced backwards action potential of the post-synaptic neuron at the synapse, resulting in facilitation. Whilst our stimulation intensity is sub-threshold (i.e. not capable of inducing forward or backward propagating action potentials), similar mechanisms may be involved. In pyramidal neurons, action potentials can be initiated with weak synaptic input provided it coincides within a few milliseconds of backwards propagating action potentials (Larkum et al., 1999). Therefore LI-rTMS may induce spike timing-dependant like plasticity by pairing weak LI-rTMS induced synaptic stimulation with post-synaptic dendritic spikes (e.g. sub-threshold calcium, sodium and NMDA dendritic spikes) or spontaneous backward propagating action potentials to increase neural activity, resulting in a transient increase in motor performance. Alternatively, priming the motor cortex with low-intensity iTBS may have transiently increased cortical excitability (Tang et al., 2016) to facilitate increased motor performance.

To further investigate the effect of low intensity priming iTBS on skilled motor behaviour, we examined whether the effect of priming iTBS was dependant on the animals inherent learning ability (i.e. whether “fast learners” were affected by priming
iTBS differently to the “intermediate” and “slow” learners”). Our results showed a trend for a greater priming iTBS effect on “intermediate” and “fast” learners, with a relatively small effect on “slow” learners. Therefore an individual’s intrinsic learning ability may limit the extent of priming iTBS-induced plasticity which would have a significant impact on the use of iTBS in motor rehabilitation and may be a factor for inter-individual variability in response to iTBS. However, further investigation is needed due to the low and unequal sample sizes in the different learning groups in our study.

To investigate the potential biochemical and molecular mechanisms underlying the priming iTBS-induced plasticity on behaviour, we conducted western blot and ELISA analysis on the stimulated cortex. Unfortunately, technical difficulties with western blotting reduced the sample size of our markers. Nevertheless, our results showed no long lasting changes in the expression of excitatory synaptic markers (GluR1, GluR2, PSD95), the inhibitory marker gephyrin, growth markers (Gap43) or in the expression of BDNF. As discussed above, our behavioural results showed that chronic priming iTBS resulted in a transient change in motor performance rather than a cumulative effect. Therefore it may not be surprising that our molecular and biochemical analysis showed no long lasting changes in mRNA or protein when assayed 24 hours after the last stimulation and training session. Alternatively, it is possible that we did not detect changes in gene or protein expression because our methods were purely quantitative and did not examine changes in protein or RNA distribution within neurons. The importance of subcellular protein trafficking in mediating HI-rTMS effects has been recently shown by altered distribution of gephyrin protein clusters, a postsynaptic marker of inhibitory synapses in hippocampal cultures following 10Hz repetitive magnetic stimulation(rMS) (Lenz et al., 2016). Interestingly, changes in gephyrin cluster size and number were not accompanied by changes in the amount of gephyrin mRNA or protein expression (Lenz et al., 2016). Therefore, the plasticity induced by priming iTBS in our study may be underpinned by mechanisms similar to those known to occur following 10Hz rMS in vitro, and involve changes in protein trafficking and distribution rather than changes in the amount of expression.

Future studies should investigate the differences and the extent of iTBS-induced neural plasticity with high and low intensities. The use of a custom rodent-specific coil
allowed for focal stimulation of the motor cortex contralateral to the dominant forepaw and avoided direct stimulation of the ipsilateral motor cortex and other cortical regions such as the cerebellum, known to influence motor learning in humans (Hardwick et al., 2013) and rodents (Nguyen-Vu et al., 2013). However a current limitation of small coils is the inability to stimulate at high intensities due to high mechanical instability and heat generated (Cohen and Cuffin, 1991). Therefore future HI-rTMS studies in mice may be better done with intracortical electrical stimulation as a proxy for rTMS (Barry et al., 2014) or in rats, in which a higher degree of focality can be achieved.

In conclusion, our results show that low intensity 120mT iTBS can be used to improve performance on a skilled motor task, resulting in a transient improvement in accuracy. Priming iTBS did not alter the rate of motor learning and was not accompanied by changes in various synaptic markers or BDNF expression. When given as a consolidating stimulus, iTBS did not alter absolute performance or the rate of motor learning compared to sham. These results highlight the importance of the timing of rTMS when modulating motor learning and demonstrate that rTMS at low-intensities is capable of inducing neural plasticity at the behavioural level.
Chapter 5: Low-intensity repetitive magnetic stimulation lowers action potential threshold and increases spike firing in layer 5 pyramidal neurons in vitro.


In chapter 4, we showed that LI-rTMS was capable of modulating motor behaviour in mice that underwent motor training in a 20 minute period after LI-rTMS. One proposed mechanism was that LI-rTMS increased neuronal excitability which led to the transient increase in motor performance. This chapter will investigate whether LI-rTMS alters neural excitability by examining potential changes in the passive and active membrane properties of layer 5 cortical pyramidal neurons from the motor and somatosensory cortex. We investigate the changes in these electrophysiological properties over the 20 minutes post-stimulation with 120mT repetitive magnetic stimulation (rMS) delivered at the iTBS frequency, as in chapter 4.

Introduction

Repetitive transcranial magnetic stimulation (rTMS) is a popular form of non-invasive brain stimulation used to induce neural plasticity in both clinical and non-clinical populations. In humans, rTMS-induced plasticity is commonly assessed at the network level by measuring the amplitude of motor evoked potentials (MEPs) elicited by single pulses of suprathreshold TMS: a change in MEP amplitude is considered to reflect change in corticospinal excitability. The onset of rTMS-induced changes in corticospinal excitability occurs immediately after stimulation and the effects persist for minutes to hours after stimulation (Huang et al., 2005, Ziemann et al., 2008, Wischnewski and Schutter, 2015). However despite widespread use in humans, and considerable evidence for changes in corticospinal excitability, the biological mechanisms underlying rTMS-induced plasticity remain unclear (Tang et al., 2015c).

Experimental models of repetitive magnetic stimulation (rMS) using organotypic tissue cultures or brain slices from animals provide a useful adjunct to human studies as they
allow direct measurement of plasticity at the single cell level and provide insights into the cellular mechanisms underlying rMS-induced plasticity (for review see references (Müller-Dahlhaus and Vlachos, 2013, Tang et al., 2015c). Recent rMS studies of subcortical neurons (organotypic hippocampal cultures) have shown that rMS induces structural and functional plasticity at inhibitory and excitatory synapses that appears 2 hours after stimulation (Vlachos et al., 2012, Lenz et al., 2014, Lenz et al., 2016).

Similarly, single cell electrophysiological studies on brain slices of rats that received rTMS show changes in the resting membrane potential and evoked spike firing of layer 2/3 fast spiking interneurons two hours after stimulation (Hoppenrath et al., 2016). However the acute effects of rMS (i.e. immediately after stimulation) on the electrophysiological properties of single cortical excitatory neurons are unknown. To investigate these effects, we employed in-vitro whole cell patch clamp electrophysiology on layer 5 pyramidal neurons from mouse motor and somatosensory brain slices. We investigated both passive and active membrane properties and evoked spiking properties following rMS or sham stimulation over a 20 minute period post-stimulation.

Methods

Ethics approval

All procedures were approved by the University of Western Australia animal ethics committee (RA/3/100/1229) which is in accordance with the Australian code of practice for the care and use of animals for scientific purposes.

Slice preparation

C57Bl/6J mice (post-natal days 12-15, of either sex, n=11) were acquired from the Animal Resource Centre (Murdoch, Australia). Mice were terminally anaesthetised with an intra-peritoneal injection of pentabarbitone (>160mg/kg) followed by rapid dissection of the brain. Acute brain slices (300µm thick) were prepared from the motor and somatosensory cortex. Coronal slices of cortex were prepared with a vibrating slicer (Campden Instruments 5000-mz) and ice-cold cutting solution comprising (mM) 125 NaCl, 3 KCl, 0.5 CaCl₂, 6 MgCl₂, 25 NaHCO₃, 1.25 NaH₂PO₄ and 10 glucose bubbled with carbogen (5% CO₂/95% O₂). Slices were kept at 35°C for 1 hour in a holding
chamber containing carbogen-bubbled artificial CSF (ACSF, see below for composition), after which they were held at room temperature until required.

**Electrophysiology**

Slices received continuous perfusion (~1.5mL/minute) with ACSF comprising (mM) 125 NaCl, 3 KCl, 2 CaCl$_2$, 1 MgCl$_2$, 25 NaHCO$_3$, 1.25 NaH$_2$PO$_4$ and 25 glucose bubbled with carbogen and maintained at 35±2°C (Warner Instruments TC-324B). For whole-cell current clamp recordings 6-10MΩ borosilicate glass patch electrodes (Harvard apparatus GC150F-15, 1.5mm outer diameter x 0.86 inner diameter, SDR scientific, Australia) were filled with an internal solution comprising (mM) 135 potassium gluconate, 10 HEPES, 7 NaCl, 2 Na$_2$ATP, 0.3 Na$_3$GTP, 2 MgCl$_2$.

Slices were visualised at 40x magnification under bright field and infrared differential interference contrast video microscopy (Olympus BX51-WI). Somatic recordings were made using a Multiclamp 700B (Axon Instruments) and digitised with a Digidata 1440A, under the control of Axograph (Axograph X 1.5.4) and data acquired at a sampling rate of 50kHz.

Whole-cell current clamp recordings were conducted on the somatic compartment of layer 5 (L5) pyramidal neurons. As we hypothesised *a priori* that stimulation would alter neuronal excitability and membrane properties, whole cell recordings were made without applying holding currents during experimental procedures.

To investigate action potential (AP) properties (AP threshold, spike rise time, spike height, fast after hyperpolarisation), single AP’s were evoked with a 5ms-long depolarising current step of +800pA (figure 5.1D), repeated every second for a total of 10s (i.e. 10 single AP’s per recording).

To investigate the spike firing properties, spikes were evoked with an AP family protocol (figure 5.1C) consisting of 500ms current steps ranging from -200 to +500pA (20 current steps, with a 30 second interstep interval), which was repeated once more after a 30s delay.

Series resistance was monitored online by briefly switching to voltage clamp, to conduct the membrane test seal function. Cells were discarded and excluded from analysis if the series resistance changed by >20% of baseline value and/or exceeded
30MΩ. Current clamp bridge balance was adjusted prior to each AP family and single AP recording.

**Repetitive magnetic stimulation (rMS)**

The rMS protocol delivered was iTBS (Huang et al 2005) (figure 5.1B) and consisted of trains of three 50Hz pulses, repeated every 200ms for 2s. Trains were repeated once every 10 seconds for a total of 20 repetitions (i.e. 600 pulses in total lasting 190 seconds). Monophasic pulses (400µs rise time) were delivered with a custom circular coil (Tang et al., 2015a) (8mm diameter, 780 turns of 0.125mm insulated copper wire around an iron-core). Coils were fixed to an electronic micro-manipulator and positioned in between the slice chamber and microscope condenser (figure 5.1A). The coil was placed at a distance of approximately 1mm from the slice such that the cortical layers were above the windings of the coil; therefore apical dendrites from layer 5 pyramidal neurons were oriented perpendicular to the coil. The peak magnetic field at a distance of 1mm from the base of the coil was measured with a Hall-effect probe (Honeywell, SS94A2D, USA) to be 85.4mT and had a dB/dT of ~285T/s. Coils were controlled by an arbitrary waveform generator (Agilent 33551B, Measurement innovation, Australia) and a programmable DC power supply (Kepco BOP 100-4M, TMG test equipment, Australia). Sham stimulation consisted of placing a coil detached from the power supply beneath the slice as described above for 190 seconds before beginning the post-stimulation recordings.

To confirm that LI-rMS did not trigger action potential firing with direct activation or through inducing suprathreshold currents in the electrode wire (Mueller et al., 2014, Pashut et al., 2014), we analysed the membrane potential recorded during LI-rMS. There was no evidence of membrane after hyperpolarisation following single or trains of LI-rMS pulses in any of the traces. Furthermore, the stimulus artefact had a peak voltage of 58.02mV ± 0.07 relative to the resting membrane potential, which is below the mean action potential spike height (~110mV, see Appendix table 1) suggesting that LI-rMS did not induce action potential firing.

**Data analysis**

Three single AP traces at each time point were analysed for (i) AP threshold (change in membrane potential at a rate of 50V/s) (ii) Spike height above resting membrane
potential (iii) 20-80% rise time of spike peak (iv) spike half-width and (v) peak fast after hyperpolarisation (AHP). AP family recordings were analysed for (i) average resting membrane potential (RMP) (ii) rheobase (lowest current step that induced 1 or more APs), (iii) input resistance (iv) spike frequency. For spike frequency analysis, the number of spikes evoked by the 500ms depolarising current steps was quantified.

**Statistical analysis**
Statistical analysis was completed with IBM SPSS statistics 20 and data graphed with Graphpad Prism 6. Only cells contributing data for each of the 4 time points were included in the analysis. Initial analyses were completed on the raw values (Appendix table 1) and confirmed that there were no significant differences in the baseline values between iTBS and sham for all the outcome measures. Further analysis on the raw values showed that although the mean baseline values were not significantly different, small differences in the baseline means obscured the detection of significant differences when running *post-hoc* tests (summary of raw values and p values provided in Appendix tables 1 and 2 respectively). Therefore, we analysed the data using an internal control method where the data at each of the post-stimulation time points was expressed as a *change relative to baseline for that cell* to account for any small differences in the baseline means between groups (Appendix table 2).

Normality was verified with Q-Q plots and homogeneity of variance tested with Levene’s test. Data were analysed with repeated measures ANOVAs. Degrees of freedom were corrected with Greenhouse-Geisser estimates when the assumptions of sphericity were violated (Mauchly’s test). *Post-hoc* testing was performed using Sidak-corrected multiple comparisons tests and p values less than 0.05 were considered statistically significant. All data are represented as mean ± standard error of the mean.
Results

rMS induces a hyperpolarised AP threshold and increases spike firing frequency

rMS induced a more hyperpolarised AP threshold (figure 5.2A) relative to sham stimulation ($F_{1,17} = 4.52$, $p = 0.048$). rMS reduced the mean AP threshold by $1.76 \text{mV} \pm 1.13$ (post-stimulation$_{+0}$), $0.77 \text{mV} \pm 1.10$ (post-stimulation$_{+10}$) and $2.08 \text{mV} \pm 1.60$ (post-stimulation$_{+20}$). Post-hoc analysis showed a significant AP threshold hyperpolarisation with rMS at post-stimulation$_{+0}$ ($p = 0.025$) and post-stimulation$_{+20}$ ($p = 0.045$).

Similarly, rMS increased evoked spike firing frequency relative to sham stimulation ($F_{1,238} = 14.813$, $p = 0.001$) (figure 5.2B). rMS increased the mean spike-firing frequency by $1.74 \text{Hz} \pm 0.32$ (post-stimulation$_{+0}$), $3.33 \text{Hz} \pm 0.54$ (post-stimulation$_{+10}$) and $4.44 \text{Hz} \pm 0.45$ (post-stimulation$_{+20}$).
0.61 (post-stimulation +20). Post-hoc analysis showed a significant increase in spike firing induced by rMS at post-stimulation +0 ($F_{1,264} = 20.681, p = 0.001$) post-stimulation +10 ($F_{1,264} = 18.781, p = 0.001$) and post-stimulation +20 ($F_{1,264} = 5.683, p = 0.018$) (Figure 5.3).

Figure 5.2. LI-rMS alters AP threshold and spike firing frequency. LI-rMS significantly hyperpolarised the AP threshold (A) and increased spike firing frequency (B). Changes in spike frequency as function of current step amplitude at post-stimulation +0 (C), post-stimulation +10 (D), post-stimulation +20 (E). * $p<0.05$, error bars represent SEM.

*rMS does not alter passive membrane properties, spike shape properties or fast after-hyperpolarisation*

In contrast to AP threshold and spike firing frequency, rMS did not significantly change RMP ($F_{1,17} = 0.56, p = 0.46$), rheobase ($F_{1,17} = 1.02, p = 0.328$), spike height ($F_{1,17} = 0.54, p = 0.473$), spike rise time ($F_{1,17} = 0.001, p = 0.983$), spike half width ($F_{1,17} = 2.320, p = 0.146$) or fast AHP ($F_{1,17} = 0.848, p = 0.370$) (figure 5.3).
rMS did not alter the input resistance \( (F_{1,17} = 0.50, p = 0.49) \) and was similar to sham for the first 10 minutes post-stimulation but trended towards a difference at 20 minutes post-stimulation (figure 5.3B). However, follow up analysis of the input resistance showed no significant differences in input resistance between sham and rMS at post-stimulation \( _{+10} \) \( (p=0.608) \), post-stimulation \( _{+10} \) \( (p=0.572) \) and post-stimulation \( _{+20} \) \( (p=0.106) \).

Figure 5.3. LI-rMS does not alter passive membrane properties, AP shape or the rheobase. LI-rMS does not alter RMP (A), input resistance (B), spike height (C), AP rise time (D), AP half width (E), fast AHP (F) or rheobase (G) \( (p>0.05) \). Error bars represent SEM.
Discussion

To our knowledge, this is the first study to investigate the acute effects of LI-rMS at the single cell level in cortical excitatory neurons. The main findings of our study show that LI-rMS does not alter passive membrane properties (RMP and input resistance) but increases neuronal excitability by inducing a more hyperpolarised AP threshold and increased evoked spike firing frequency relative to sham stimulation. Changes in AP threshold were present immediately after and 20 minutes post-stimulation whereas spike frequency changes were found immediately after stimulation and persisted to 20 minutes post-stimulation.

Given that the RMP remained unchanged, a hyperpolarised AP threshold is evidence of an LI-rMS-induced increase in neuronal excitability, due to modulation of membrane potential mechanisms at depolarised levels. Mechanisms affecting AP threshold/AP initiation include changes in fibre thickness and in the density and properties of voltage gated sodium channels (Kole et al., 2008, Stuart et al., 1997). However, the changes in AP threshold were observed immediately after stimulation (190 seconds after onset), and changes in fibre thickness and the density of voltage gated sodium channels (VGSCs) are unlikely to have occurred within such a short timeframe. Moreover, high intensity rMS-induced structural changes, including changes in receptor density, have previously been shown to take greater than two hours post-stimulation (Vlachos et al., 2012). Rather, it is more likely that LI-rMS induced changes in the properties of VGSCs, and has been suggested previously for voltage gated calcium channels (for review see reference (Pall, 2013)). Such a mechanism may underlie the change in AP threshold, probably through a direct modulation of the voltage sensing mechanism, resulting in the opening of the VGSCs at more hyperpolarised voltages. Interestingly, the changes in AP threshold were present immediately and at 20 minutes post-stimulation but were not at 10 minutes post-stimulation. The apparent cyclical nature of AP threshold hyperpolarisation may be due to LI-rMS altering the voltage sensing mechanism by two different pathways, each with different times of onset. The immediate AP threshold hyperpolarisation may be due to a direct interaction with LI-rMS (e.g. with the induced electric field during stimulation) whereas the AP threshold hyperpolarisation 20 minutes post-stimulation may be due to activation of a biochemical/signalling pathway with a longer onset. As
LI-rMS has previously been shown to increase intracellular calcium release in cortical neurons (Grehl et al., 2015), one such pathway that may underlie the longer onset change to AP threshold is through calcium signalling/calmodulin which is known to alter VGSC function (Herzog et al., 2003).

Our second line of evidence that LI-rMS increases neuronal excitability is the increase in evoked spike firing following stimulation. Multiple channels are known to regulate spike firing frequency through alterations in the after hyperpolarisation that follows spike firing (Hille, 2001), including A-type potassium channels (K_A) for L5 pyramidal neurons (Kang et al., 2000) which may have been modulated by LI-rMS. Interestingly, analysis of our single AP data revealed no change in the peak fast AHP accompanying the significant increases in spike firing frequency (see Figure 5.3F). The involvement of K_A channels varies between single and repetitive firing, with a greater role of K_A channels during repetitive firing (Kang et al., 2000). Therefore LI-rMS-induced increases in spike frequency may be due to modulation of specific K_A channel properties, which would require separate pharmacological investigation.

Since LI-rMS lowered the AP threshold, we expected to observe a concurrent reduction in the rheobase current. Whilst the mean rheobase current decreased over time following LI-rMS, it did not reach statistical significance. It is possible that our increments in current steps (25pA steps in the 50-200pA range) may have been too large to detect subtle changes in rheobase (i.e. changes <25pA) underlying a reduction in AP threshold of ~2mV.

Our results are in part, similar to a recent study using high intensity rTMS (Hoppenrath et al., 2016). The authors also show that rTMS increases neuronal excitability, with increased spike frequency in fast-spiking interneurons when probed two hours post-stimulation. Interesting the authors also found changes to the resting membrane potential. Therefore it is possible that LI-rTMS and rTMS share common effects (e.g. changes in spike frequency) with high intensity rTMS capable of more profound effects (e.g. altered resting membrane) due to increased intensity.

Recordings of the membrane potential during LI-rMS confirmed that the delivered stimulation intensity did not directly induce AP firing (i.e. subthreshold stimulation). In contrast to high intensity rTMS where stimulation is believed to result in neuronal
firing through trans-synaptic (Labedi et al., 2014, Lenz et al., 2016) or direct activation (Lenz et al., 2016). Therefore our results provide further evidence that subthreshold stimulation induced by LI-rMS is capable of modulating neural plasticity. These results are in line with previous studies from our laboratory that used 12mT rTMS in mice (approximately 2 orders of magnitude lower than suprathreshold rTMS) to induce structural and molecular plasticity (Rodger et al., 2012, Makowiecki et al., 2014). Although we provide evidence that LI-rMS modulates certain electrophysiological properties of cortical pyramidal neurons, further studies are needed to determine whether LI-rMS-induced plasticity is neuron subtype specific (e.g. pyramidal vs interneurons) or brain region specific (e.g. cerebellum (Morellini et al., 2014) vs hippocampus etc.) as well as whether non-neuronal cells such as glia (Cullen and Young, 2016) can be modulated.

It is well established that the endogenous electrophysiological properties of pyramidal neurons differ between young and adult animals (Zhang, 2004, Etherington and Williams, 2011). In our study, we used slices from developing mice (12-15 days post-natal) whereas previous high intensity rMS studies have mostly used adult animals (~3 months old) to demonstrate plasticity of both inhibitory and excitatory networks using electrophysiological (Hsieh et al., 2014, Thimm and Funke, 2015) and molecular methods (Hoppenrath and Funke, 2013, Trippe et al., 2009). Interestingly, the recent study by Hoppenrath et al showed a significant age effect on high intensity rMS-induced plasticity in fast-spiking interneurons, with increases in evoked spike firing frequency present in young adult animals (post-natal days 29-38) but absent in juvenile (post-natal days 26-28) and older adult animals (post-natal days 40-62) (Hoppenrath et al., 2016). In contrast, our results show LI-rMS alters the excitability properties (including evoked spike firing frequency) in motor and somatosensory slices from developing animals (post-natal days 12-15). At this age, pyramidal neurons are in a heightened state of plasticity as they are approaching the end of the critical periods for both motor and somatosensory systems (Hensch, 2004). The intrinsic properties of the developing neurons, including their heightened plasticity state, may affect the capacity of LI-rMS to induce plasticity and the mechanism whereby it does so. Therefore, future studies in older animals are needed to determine whether the changes observed in developing tissue also occur in adult tissue. However, we have previously shown that
LI-rTMS increases corticospinal excitability in anaesthetised adult rats, providing evidence of LI-rTMS-induced plasticity in adult animals (Tang et al., 2016).

We have provided insight into the acute effects of LI-rMS on single excitatory cortical neurons. Our results show that LI-rMS increases excitability of L5 pyramidal neurons from motor and somatosensory brain slices, by modulating specific active and spiking properties without altering passive membrane properties. These results further our understanding of LI-rMS-induced plasticity and highlight the capability of subthreshold magnetic stimulation to induce functional plasticity.
Chapter 6: General Discussion

What’s new?

This thesis aimed to characterise the effect of LI-rTMS on neuroplasticity in both healthy and neurotrauma animals and experimental models, and to provide insight into the cellular and molecular mechanisms underlying LI-rTMS-induced plasticity. Disappointingly, LI-rTMS (12mT) does not induce neuroprotective or regenerative plasticity following severe injury to the optic nerve. To address the possibility that our LI-rTMS intensity was “too low”, in subsequent chapters, I developed novel rodent-specific rTMS coils capable of delivering rTMS reliably at 120mT (although these have not yet been tested in an injury model). Using these coils, I show that LI-rTMS can modulate skilled motor behaviour and alter specific electrical properties of excitatory neurons. Taken together, the results of this thesis (1) provide insight into the cellular mechanisms underlying LI-rTMS induced plasticity, (2) provide the first rodent-specific coil capable of focal rTMS and biologically relevant intensities to rodents, and (3) challenge the current concept of what rTMS intensities are clinically relevant.

Mechanisms of low-intensity rTMS-induced plasticity

Although not fully understood, HI-rTMS is believed to induce both synaptic and non-synaptic changes that modulated plasticity (Müller-Dahlhaus and Vlachos, 2013, Tang et al., 2015c). This thesis has shown that LI-rTMS induces plasticity that shares some aspects with that induced by HI-rTMS, such as increased MEP amplitudes, enhanced motor behaviour and increased neuronal excitability. Furthermore, previous studies from our lab have suggested overlapping mechanisms between high and low intensity such as calcium increases and BDNF upregulation (Rodger et al., 2012, Makowiecki et al., 2014, Grehl et al., 2015). However, despite producing similar effects and potential overlapping mechanisms, evidence from this thesis suggests LI-rTMS induced plasticity is, at least in part, mediated by mechanisms distinct to those of HI-rTMS.

Unlike HI-rTMS, where stimulation induces suprathreshold currents and is believed to induce action potential dependent plasticity (Lenz et al., 2014), LI-rTMS induces subthreshold currents. It is therefore intuitive that LI-rTMS plasticity mechanisms are distinct from those of HI-rTMS: as the stimulation intensity is too low to directly induce action potentials, any plasticity must be independent of induced action potentials. The
subthreshold nature of our protocols is supported by recordings of the membrane potential during LI-rMS. Even though stimulation induced a large artefact, we did not detect any evidence of action potentials. Similarly, FEM modelling of the induced electric field in a simplified rat brain undergoing LI-rTMS suggests that our coils induce a maximum electric field of 12.7 V/m which is below the range of electric fields shown to induce action potentials experimentally (30-130V/m) (Radman et al., 2009). However, we do not discount the possibility that LI-rTMS interacts with endogenous neural activity, such as dendritic spiking/post-synaptic potentials and/or spontaneous neural activity to induce plasticity.

At high magnetic field intensities, NMDA receptors are known to play a significant role in rTMS-induced plasticity. In humans, pre-treatment with memantine, an NMDA receptor antagonist abolishes the induction of rTMS-induced MEP facilitation (Huang et al., 2007). In rats, low doses of the NMDA receptor antagonist ketamine (30mg/Kg), decreased rTMS-induced reductions in inhibitory markers, whereas high doses (60mg/Kg) almost completely abolished the effect of rTMS (Labedi et al., 2014). Similarly, in hippocampal cultures, pharmacological blockade of NMDA receptors during the delivery of rMS prevents the induction of synaptic plasticity (Vlachos et al., 2012). In contrast, results from this thesis suggest that LI-rTMS induced plasticity via a non-NMDA receptor dependent mechanism. Our first line of evidence comes from the small number of rats anaesthetised with a mixture containing ketamine, a NMDA receptor antagonist at a surgical dose (50mg/Kg) prior to receiving 10Hz LI-rTMS or sham to the motor cortex. Our results showed that sham stimulation does not alter corticospinal excitability/MEP amplitude whereas active 10Hz LI-rTMS increases MEP amplitudes, even in the presence of ketamine. Our second line of evidence comes from our whole-cell patch clamp experiments where LI-rMS increased neuronal excitability. Our results showed that LI-rMS hyperpolarises the action potential threshold and increases evoked spike firing frequency. Whilst further pharmacological manipulation is needed to determine the exact cellular mechanisms involved, action potential threshold and spike firing frequency are not NMDA receptor-dependent but are instead mediated by other receptors such as voltage gated sodium (Kole et al., 2008) and potassium channels (Hille, 2001, Kang et al., 2000), further suggesting that LI-rTMS plasticity is a non-NMDA receptor dependent process.
Another distinguishing feature of LI-rTMS is the ability to induce plasticity in “young” neurons. The endogenous electrophysiological properties of pyramidal neurons differ between juvenile and adult animals (Zhang, 2004, Etherington and Williams, 2011) and this affects the capacity of HI-rMS, but to a lesser extent of LI-rTMS to induce plasticity. In an interesting study, Hoppenrath et al recently investigated the effect of high intensity iTBS on passive and active membrane properties in inhibitory fast-spiking interneurons (similar to chapter 5 of this thesis). In their study, iTBS induced neuromodulatory effects exclusively in adult animals (post-natal days 40-62) but not in young or old animals (post-natal days 26-28 and 40-62 respectively) (Hoppenrath et al., 2016). In contrast, we found neuromodulatory effects of LI-rMS on brain slices from juvenile animals (post-natal days 12-15). Several differences (aside from intensity) exist between our study and that of Hoppenrath et al such as the temperature at which recordings were made (room temperature vs 35˚C in our study) and the time at which changes in plasticity were probed post-stimulation (2hrs post-stimulation vs. 0-20 minutes post-stimulation in our study) which may explain why we found changes in young animals. However, given that we did observe neuromodulatory effects of LI-rMS in young brain slices and that LI-rTMS also induced plasticity in vivo in adult rats and mice (chapters 3 and 4 respectively), we suggest that LI-rTMS is capable of inducing plasticity in both young and adult nervous systems. From a clinical perspective, the results from this thesis warrant further investigation into the effect of LI-rTMS on young tissue, as rTMS is used to treat neuropsychiatric disorders in children using adult rTMS parameters. As these protocols have been developed and tested in adults, more work is needed to determine whether they are suitable and optimal for children (Croarkin et al., 2010).

Low-intensity rTMS-induced plasticity may be cell/region specific

In addition to the uncertainties surrounding the basic mechanisms of rTMS-induced plasticity, there are unanswered questions about whether all cell types of the nervous system (e.g. neurons and glia) are modulated with rTMS and whether different brain regions show different responses. Studies from our lab have previously shown that LI-rTMS to the visual pathway of mice increases the local concentration of BDNF in the visual cortex and superior colliculus (Rodger et al., 2012, Makowiecki et al., 2014). However, when applied directly over the orbit of the eye to stimulate RGCs, LI-rTMS
did not alter BDNF concentration in the retina or optic nerve of intact mice or mice that received an optic nerve crush, suggesting that LI-rTMS modulation of BDNF may be cell type specific, at least in the visual pathway. This is in line with BDNF upregulation in the hippocampus with HI-rTMS, with increases observed in specific hippocampal regions (CA3) but not throughout all hippocampal regions (CA1 and CA2) (Müller et al., 2000).

Similarly, I showed that LI-rTMS modulated spike firing frequency in layer 5 pyramidal neurons (Chapter 5). Repetitive spike firing in cortical pyramidal neurons is controlled by $K_A$ channels (Kang et al., 2000, Hille, 2001), but in other brain regions different potassium channel subtypes are expressed in different neuron subtypes (e.g. BK channels in CA1 pyramidal neurons (Gu et al., 2007) and SK channels in striatal interneurons (Orduz et al., 2013). While further pharmacological investigation is needed to determine the exact mechanisms/channels underlying LI-rTMS increases in evoked spike firing frequency, potassium channel subtype may contribute or even determine the outcome of LI-rTMS changes in neuronal excitability in different brain regions.

**Delivering focal rTMS to rodents**

Despite the advantages of studying rTMS in rodents, directly translating results from rodents to humans is difficult as the commercially available coils stimulate multiple brain regions if not the entire brain and part of the spinal cord, making generalisations to focal cortical stimulation in humans invalid (Wassermann and Zimmermann, 2012). Downsizing coils to improve focality is technically challenging as the large currents required to generate the large magnetic fields (e.g. 1T) to replicate human rTMS increases mechanical and thermal stress inside the coil (Cohen and Cuffin, 1991). In chapter 3, we designed and characterised two novel coils that we hoped would bridge the gap between the use of 12mT rodent-specific coils and the use of 1T commercial coils. Whilst we achieved our primary aim of increasing the magnetic field intensity from 12mT to 120mT, these coils still do not replicate the high intensities generated with commercial coils. It should be noted that while the ultimate goal of rodent coils is to produce a small coil which delivers both focal and high intensity magnetic fields, this may not be possible as increased focality is at the expense of intensity and vice versa, which has been well characterised in human rTMS coils (Deng et al., 2013). It seems
more likely that the future choice of coils for investigators using rodent models will come down to prioritising either focality or intensity, or using focal electrical stimulation as a proxy for rTMS (Barry et al., 2014).

Given that small human coils can be used to achieve some level of focality and induce suprathreshold stimulation unilaterally (Rotenberg et al., 2010) and the recent commercialisation of a “rat-specific” coil capable of inducing MEPs with a cooling attachment to minimise thermal stress (Parthoens et al., 2016), do our novel 8mm coils offer any advantage to their commercial counterparts? We argue that when focality is the priority, our coils offer great spatial resolution as evident from our electric field modelling in a simplified rat brain. Comparison with the commercial Magventure butterfly coil which we used to induce MEPs, shows that this human coil stimulates the entire rat brain (chapter 3). Experimentally, this was shown in the seminal rodent MEP study which outlined the method using an offset figure of 8 coil, where bilateral activation occurs at higher intensities (e.g. 90-100% machine stimulator output) (Rotenberg et al., 2010) and provides further evidence that the induced electric field by the figure of 8 coil is still relatively unfocal. Similarly, the “rat-specific” coil by Parthoens et al, does not address the issue of focality. In testing their rat coil, the authors demonstrate that that the coil results in unfocal stimulation as placement of their coil over the cortex and towards the spinal cord did not result in different MEP latencies. Instead, they found similar MEP latencies regardless of where the coil was placed (Parthoens et al., 2016), indicating a large induced electric/magnetic field that encapsulates the entire brain and parts of the spinal cord. In contrast, electric field modelling of our novel coils in a rat brain suggests that our coils induce focal electric fields and can be used to stimulate unilaterally, with stimulation restricted to within a few millimetres of the peak electric field underneath the windings of the 8mm coil. Therefore, although our coils do not permit single pulse MEP style studies in rats, they allow the investigation of LI-rTMS on specific brain regions which is not currently possible with commercial coils (i.e. the effect of LI-rTMS on the motor cortex vs effect on the cerebellum).

Is low-intensity stimulation clinically irrelevant?
As mentioned in chapter 1, LI-rTMS is often considered clinically irrelevant as the induced electric field/ electrical currents are an order of magnitude lower than clinical
high-intensity rTMS and do not induce action potentials. However, evidence from this thesis in addition to previous *in vivo* and *in vitro* work from our lab and others challenge this assumption and show that subthreshold repetitive magnetic fields have a profound effect on neurons. Specifically in this thesis, I have shown that ~100mT stimulation alters neural plasticity at the single cell level and extends to the behavioural level.

Similar to high intensity stimulation in humans, I have shown that LI-rTMS can alter motor behaviour in mice. At high intensities, a single session of priming rTMS increases performance improvement across trials (i.e. learning) (Teo et al., 2011) whereas daily sessions of priming rTMS combined with motor learning has a detrimental effect, whereby the rTMS group shows decreased motor learning (Läppchen et al., 2015) relative to sham, possibly due to the recruitment of metaplastic mechanisms. In contrast, our results show that daily priming skilled motor learning with LI-rTMS has therapeutic potential as it enhances daily performance (increased accuracy) but does not alter the rate at which they learn a task over 10 consecutive days relative to sham stimulation. We suggest that an advantage to using LI-rTMS versus HI-rTMS in the clinical setting is that LI-rTMS can induce a positive effect on motor behaviour without recruiting metaplastic mechanisms, which may have important clinical implications.

Similarly, our investigations *in vitro* show that low intensity stimulation is capable of altering neural excitability. HI-rTMS, particularly with iTBS, is routinely used to modulate excitability of the motor cortex at the network level (Chung et al., 2016). At the single cell level, high intensity iTBS has been shown to depolarise the resting membrane potential and increases the evoked spike firing rate of cortical fast-spiking interneurons when probed two hours after stimulation (Hoppenrath et al., 2016). We provide evidence that low-intensity iTBS increases evoked spike firing rate of excitatory pyramidal neurons and hyperpolarises the action potential threshold that occur within 20 minutes post-stimulation, similar to the time course of changes in corticospinal excitability at the network level seen in humans with high intensity stimulation (Wischnewski and Schutter, 2015). Therefore these results provide further evidence that low intensity stimulation induces neuromodulatory effects and should not be considered as clinically irrelevant.
However, this thesis also highlights the clinical limitations of LI-rTMS. In addition to investigating plasticity in the intact nervous system, I examined whether LI-rTMS could be used to directly treat severe neurotrauma (i.e. as a treatment for cell death and axon regeneration rather than as an adjunct for rehabilitation). We have previously shown that at 12mT, LI-rTMS can induce collateral sprouting from surviving neurons in a partial injury model in mice (Morellini et al., 2014). Using the same LI-rTMS protocol in a complete optic nerve crush to induce RGC cell death and axon degeneration, we show that LI-rTMS at 12mT does not induce neuroprotection or axon regeneration in adult animals. These results suggest that LI-rTMS may have therapeutic potential in the treatment of partial/less severe injuries, but does not hold therapeutic potential/clinical relevance for severe neurotrauma. It remains to be determined whether the outcome could be improved by using the novel rodent coil to deliver 120mT instead of 12mT.

Limitations of rodent and experimental models compared to human TMS

Intensity aside, there are several key differences in rodent and human TMS. A key difference between our rodent studies and human TMS is the use of anaesthesia and manual restraint when delivering rTMS. Rat MEPs were conducted under anaesthesia in order to avoid injury and distress to the animals and to obtain “clean” EMG traces. Although it might seem counterproductive to suppress CNS excitability and activity when studying neuromodulatory interventions, rTMS plasticity can be induced reliably in anaesthetised animals (Gersner et al., 2011, Muller et al., 2014). Nonetheless, we acknowledge the limitations of anaesthesia as there is evidence for differences in synaptic and biochemical changes following rTMS in anaesthetised compared to awake rats (Gersner et al., 2011). When studying LI-rTMS and motor behaviour, LI-rTMS was delivered to lightly restrained mice. The advantage of mechanical restraint over anaesthesia is the delivery of rTMS to awake animals which maintains spontaneous neural activity. However mechanical restraint is likely to induce stress and upregulate cortisol, which has been shown to alter cortical excitability of both excitatory and inhibitory circuits (Milani et al., 2010) and may have affected the direction and magnitude of LI-rTMS induced plasticity.
A further consideration is that in contrast to humans, rats and mice show very little cortical folding and have smooth cortices. Cortical folding is known to influence the induced electric field pattern across the cortex (Thielscher et al., 2011). As rodents have smooth cortices, the induced electric field is distributed more evenly across the cortex relative to humans. Similarly, all animals used in this thesis are genetically homogenous and are identically housed. Although not the theme of this thesis, anatomical and genetic differences are suggested to influence rTMS plasticity and inter-subject variability in humans (Ridding and Ziemann, 2010) and these complex factors cannot be easily reproduced or modelled in rodents.

Even further removed from human rTMS, we used rodent brain slices to characterise the effect of LI-rTMS at the single cell level. Brain slice electrophysiology has been used extensively to determine basic neural functions, plasticity and to determine the mechanisms of non-invasive brain stimulation (Radman et al., 2009, Bikson et al., 2004, Pashut et al., 2014). Unlike in vivo electrophysiology, in brain slices, cells from all cortical layers and sub-cortical regions can be accessed but there are also limitations. The main limitation of brain slice preparations is the reduction in network and spontaneous activity as a result of preparing brain slices where the majority of afferent inputs are severed (Radman et al., 2009). Given that spontaneous neural activity (Gersner et al., 2011) and co-operation between pre and postsynaptic neurons (i.e. spike timing dependent plasticity) (Thickbroom, 2007, Lenz et al., 2014) are potential mechanisms underlying rTMS-induced plasticity, our changes in neuronal excitability in vitro may not be representative of the changes seen in vivo.

**Conclusion**

In summary, this thesis provides strong evidence that LI-rTMS has a profound effect on neural plasticity both at the single cell and behavioural level. Moreover, I have developed novel rodent-specific rTMS coils capable of focal stimulation and characterised the effect of LI-rTMS on both the healthy and intact nervous system. The experiments in this thesis have provided mechanistic insights into how LI-rTMS induces plasticity and suggests that regions outside the targeted region with HI-rTMS are susceptible to neuromodulation which may contribute to the overall plasticity induced by rTMS. I hope that the results of this thesis and future investigations of the molecular and cellular mechanisms underlying both low and high intensity will pave
the way for optimising rTMS at a range of intensities in the treatment of neurological disease and disorders.
References


dynamic motor learning is not disrupted by rTMS of primary motor cortex.
Current Biology, 14, 252-256.

of conduction time in measurements in central motor pathways using magnetic


BARRY, M. D., BODDINGTON, L. J., IGELSTRÖM, K. M., GRAY, J. P., SHEMMELL, J.,
theta burst electrical stimulation to attenuate interhemispheric inhibition and
to promote motor recovery after cortical injury in an animal model.
Experimental Neurology, 261, 258-266.

BATES, D., MAECHLER, M., BOLKER, B. & WALKER, S. 2014. lme4: Linear mixed-effects

BATES, K. A., CLARK, V. W., MELONI, B. P., DUNLOP, S. A. & RODGER, J. 2012. Short-
term low intensity PMF does not improve functional or histological outcomes in
a rat model of transient focal cerebral ischemia. Brain Research, 1458, 76-85.

synapse-specific homeostatic plasticity. Proceedings of the National Academy

nerve regeneration. Experimental Neurology.


in delayed death and apoptosis of retinal ganglion cells in adult rats. Journal of
Neuroscience, 14, 4368-4374.

BICKFORD, R. & FEMMING, B. Neural stimulation by pulsed magnetic fields in animals
and man. 6th International Conference on Medical Biological Engineering.
Tokyo, paper, 1965. 7-6.

BIKSON, M., INOUE, M., AKIYAMA, H., DEANS, J. K., FOX, J. E., MIYAKAWA, H. &
JEFFERYS, J. G. R. 2004. Effects of uniform extracellular DC electric fields on


of neurons in the retinal ganglion cell layer in relation to foraging behaviors of tyrant flycatchers. *Journal of Comparative Neurology*, 514, 66-73.


protein expression in the rat hippocampus impairs the maintenance of long-term potentiation and the consolidation of long-term memory. *Journal of Neuroscience*, 20, 3993-4001.


transduction of injury signals after unilateral optic nerve crush. *NeuroReport*, 20, 301-305.


PALL, M. L. 2013. Electromagnetic fields act via activation of voltage-gated calcium channels to produce beneficial or adverse effects. Journal of Cellular and Molecular Medicine, 17, 958-965.


Repetitive Transcranial Magnetic Stimulation Coil for the Rat. 

PASCUAL-LEONE, A. 1999. Transcranial magnetic stimulation: studying the brain--
behaviour relationship by induction of ‘virtual lesions’. *Philosophical
Transactions of the Royal Society of London B: Biological Sciences*, 354, 1229-1238.


PASHUT, T., MAGIDOV, D., BEN-PORAT, H., WOLFUS, S., FRIEDMAN, A., PEREL, E.,
recordings of rat neurons from acute brain slices of the somatosensory cortex

PASHUT, T., WOLFUS, S., FRIEDMAN, A., LAVIDOR, M., BAR-GAD, I., YESHURUN, Y. &

PAULUS, W. & ROTHWELL, J. C. 2016. Membrane resistance and shunting inhibition:
where biophysics meets state-dependent human neurophysiology. *Journal of
Physiology*, 594, 2719-2728.

New York, Academic Press Inc.

PEINEMANN, A., REIMER, B., LÖER, C., QUARTARONE, A., MÜNCHAU, A., CONRAD, B. &
ROMAN SIEBNER, H. 2004. Long-lasting increase in corticospinal excitability
after 1800 pulses of subthreshold 5 Hz repetitive TMS to the primary motor

PELLETIER, S. J. & CICCHETTI, F. 2015. Cellular and molecular mechanisms of action of
transcranial direct current stimulation: evidence from in vitro and in vivo
models. *International Journal of Neuropsychopharmacology*, 18, pyu047.

PINHEIRO, J., BATES, D., DEBROY, S., SARKAR, D., HEISTERKAMP, S., VAN WILLIGEN, B.

Neuroscience*, 2, 24-32.


or a Caspase 3 Inhibitor RGC Loss and Caspase 3 Activation After Axotomy in Mice. *Investigative Ophthalmology and Visual Science*, 57, 81-93.


### Table 1. Summary table of raw values for chapter 5

<table>
<thead>
<tr>
<th>Membrane property</th>
<th>Stimulation</th>
<th>Baseline</th>
<th>Post-stimulation&lt;sub&gt;-0&lt;/sub&gt;</th>
<th>Post-stimulation&lt;sub&gt;+10&lt;/sub&gt;</th>
<th>Post-stimulation&lt;sub&gt;+20&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membrane potential (mV)</td>
<td>Sham</td>
<td>-65.0±1.4</td>
<td>-64.9±1.4</td>
<td>-64.6±1.4</td>
<td>-63.5±1.4</td>
</tr>
<tr>
<td></td>
<td>iTBS</td>
<td>-64.8±1.5</td>
<td>-63.9±1.6</td>
<td>-62.7±1.4</td>
<td>-62.9±1.5</td>
</tr>
<tr>
<td>Input resistance (MΩ)</td>
<td>Sham</td>
<td>91.1±11.3</td>
<td>93.8±11.9</td>
<td>90.3±9.4</td>
<td>96.3±11.5</td>
</tr>
<tr>
<td></td>
<td>iTBS</td>
<td>93.5±11.9</td>
<td>98.2±12.5</td>
<td>95.1±9.9</td>
<td>86.5±12.1</td>
</tr>
<tr>
<td>AP threshold (mV)</td>
<td>Sham</td>
<td>-42.8±1.8</td>
<td>-40.8±1.8</td>
<td>-41.9±1.9</td>
<td>-40.1±2.1</td>
</tr>
<tr>
<td></td>
<td>iTBS</td>
<td>-41.5±1.9</td>
<td>-43.3±1.9</td>
<td>-42.3±1.9</td>
<td>-43.6±2.2</td>
</tr>
<tr>
<td>Rheobase (pA)</td>
<td>Sham</td>
<td>147.5±19.6</td>
<td>150.0±19.4</td>
<td>150.0±17.8</td>
<td>130.0±14.4</td>
</tr>
<tr>
<td></td>
<td>iTBS</td>
<td>144.4±20.7</td>
<td>133.3±20.4</td>
<td>119.4±18.7</td>
<td>122.2±15.2</td>
</tr>
<tr>
<td>Spike height (mV)</td>
<td>Sham</td>
<td>112.5±1.5</td>
<td>112.5±1.5</td>
<td>110.1±1.9</td>
<td>109.8±1.9</td>
</tr>
<tr>
<td></td>
<td>iTBS</td>
<td>111.6±1.6</td>
<td>111.0±1.7</td>
<td>107.8±2.0</td>
<td>106.0±2.0</td>
</tr>
<tr>
<td>20-80% AP rise time (ms)</td>
<td>Sham</td>
<td>0.23±0.06</td>
<td>0.29±0.12</td>
<td>0.27±0.07</td>
<td>0.43±0.25</td>
</tr>
<tr>
<td></td>
<td>iTBS</td>
<td>0.29±0.07</td>
<td>0.34±0.13</td>
<td>0.28±0.08</td>
<td>0.53±0.26</td>
</tr>
<tr>
<td>AP half width (ms)</td>
<td>Sham</td>
<td>0.79±0.06</td>
<td>0.75±0.06</td>
<td>0.78±0.06</td>
<td>0.78±0.06</td>
</tr>
<tr>
<td></td>
<td>iTBS</td>
<td>0.83±0.07</td>
<td>0.85±0.07</td>
<td>0.88±0.07</td>
<td>0.88±0.07</td>
</tr>
<tr>
<td>Fast AHP peak (mV)</td>
<td>Sham</td>
<td>-1.97±0.49</td>
<td>-1.75±0.42</td>
<td>-1.77±0.49</td>
<td>-1.73±0.44</td>
</tr>
<tr>
<td></td>
<td>iTBS</td>
<td>-2.27±0.52</td>
<td>-1.70±0.44</td>
<td>-1.83±0.52</td>
<td>-1.88±0.46</td>
</tr>
<tr>
<td>Spike frequency (Hz)</td>
<td>Sham</td>
<td>19.29±1.59</td>
<td>19.06±1.57</td>
<td>19.44±1.6</td>
<td>21.71±1.73</td>
</tr>
<tr>
<td></td>
<td>iTBS</td>
<td>17.90±1.68</td>
<td>19.64±1.65</td>
<td>21.22±1.71</td>
<td>22.33±1.82</td>
</tr>
</tbody>
</table>
Table 2. Summary of p values from repeated measures ANOVA’s of raw values for chapter 5.

<table>
<thead>
<tr>
<th>Membrane property</th>
<th>TIME</th>
<th>STIMULATION</th>
<th>TIME X STIMULATION</th>
<th>CURRENT STEP</th>
<th>STIMULATION X CURRENT STEP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membrane potential</td>
<td>0.278</td>
<td>0.421</td>
<td>0.404</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Input resistance</td>
<td>0.391</td>
<td>0.977</td>
<td>0.077</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AP threshold</td>
<td>0.980</td>
<td>0.639</td>
<td>0.039</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Rheobase</td>
<td>0.139</td>
<td>0.552</td>
<td>0.359</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Spike Height</td>
<td>0.002</td>
<td>0.348</td>
<td>0.475</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>20-80% AP rise time</td>
<td>0.139</td>
<td>0.781</td>
<td>0.827</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AP half-width</td>
<td>0.398</td>
<td>0.334</td>
<td>0.490</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Fast AHP peak</td>
<td>0.816</td>
<td>0.441</td>
<td>0.783</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Spike frequency</td>
<td>0.001</td>
<td>0.669</td>
<td>0.001</td>
<td>0.001</td>
<td>0.99</td>
</tr>
</tbody>
</table>