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Investigation of brain stimulation in animal models of depression and Alzheimer's disease: feasibility of tailored treatments

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Investigation of brain stimulation in animal models of depression and Alzheimer's disease: feasibility of tailored treatments

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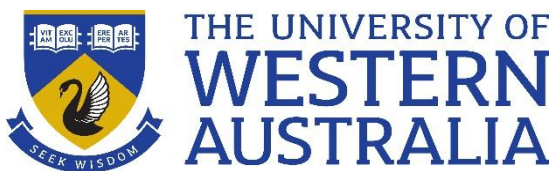
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Neuroscience Discipline

2018

Thesis Declaration

I, Alesha Heath, certify that:

This thesis has been substantially accomplished during enrolment in the degree.

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The research involving animal data reported in this thesis was assessed and approved by The University of Western Australia Animal Ethics Committee Approval #RA03/100/1298 and the Comité National d’Ethique pour les Sciences de la Vie et de la Santé N°5 (N° 1493-02).

The research involving animals reported in this thesis followed The University of Western Australia and national standards for the care and use of laboratory animals.

The work described in this thesis was funded by the Neurotrauma Research Programme

This thesis contains published work and work prepared for publication, some of which has been co-authored.

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Abstract

Repetitive transcranial magnetic stimulation (rTMS) is a form of non-invasive brain stimulation that can be used to alter the neuroplasticity of the brain and is used to treat various neurological disorders. However, there is still uncertainty about the mechanisms of action and how altering the parameters of the stimulation can affect the desired outcome. This thesis explored rTMS in two preclinical models of neurological disorders. One in which rTMS is an approved treatment; depression, and one where using rTMS is a novel therapy technique; Alzheimer's Disease (AD). These disorders both have different pathologies, so we therefore wanted to explore the possibility that rTMS can be tailored to treat these different disorders more effectively.

We first investigated the effects of rTMS in a preclinical model of depression, altering the intensity of stimulation to see how this affected the antidepressant result. Olfactory bulbectomized (OB) mice were treated with either low-intensity rTMS (LI-rTMS, 12mT), medium-intensity rTMS (MI-rTMS, 90mT) or high intensity rTMS (HI-rTMS, 1.2T) delivered at 10Hz, 5 days per week over 4 weeks, focused over the frontal cortex. The psychomotor agitation induced by OB in the forced swim test was attenuated with MI-rTMS and HI-rTMS, but only MI-rTMS increased brain-derived neurotrophic factor and neurogenesis in specific brain regions and HI-rTMS normalized plasma concentration of α -amino-n-butyric acid and 3-methylhistidine. Ingenuity Pathway Analysis revealed significant changes in glutamine processing and glutamate signalling in the OB model and following MI-rTMS and HI-rTMS treatment.

We then investigated the effect of LI-rTMS stimulation in a preclinical model of AD, altering the frequency of the stimulation. Six month and 12 month female PS1M146V mice, a genetic knock in model of AD, and C57BL6/J age-matched controls were treated with LI-rTMS focused over the hippocampus. Four different frequencies were used; two classically excitatory: Biomimetic High Frequency (BHFS) and 10Hz and two classically inhibitory: 1Hz and cTBS. After two weeks of stimulation, in the six month mice, we found positive effects of the two excitatory frequencies in restoring Morris Water Maze spatial memory deficits and dendritic spine abnormalities. In contrast, the two inhibitory frequencies had different effects, with 1Hz stimulation inducing partial improvements and cTBS having either no effect or in some cases worsening these measures. None of the stimulation protocols improved the lower neurogenesis in AD mice, but brain

derived neurotrophic factor (BDNF) expression was slightly increased by 10Hz stimulation. Importantly, the stimulations had no effect on the anxious phenotype seen in the Elevated Plus Maze, suggesting that the improvement in memory was not influenced by less anxiety. Interestingly, at 12 months though the stimulation continued to improve the memory and dendritic deficits in the AD mice, in the control mice it had a negative effect.

Comparing the results from both these studies suggests that rTMS can be tailored to specific neurological conditions by altering the location, intensity and frequency of stimulation. For a disorder such as depression where a change in limbic connectivity is an underlying cause of the disorder, using higher intensity forms of the stimulation focused over the frontal cortex proved most effective. Whereas, in AD where the ability to maintain synaptic connections is affected, LI-rTMS focused to the area of deficit to improve the underlying ability for plasticity, may be more appropriate.

Abstrait

La stimulation magnétique transcrânienne (abréviation de l'anglais : TMS) est une forme de stimulation non-invasive du cerveau qui modifie la plasticité neuronale. Sous sa forme répétitive, rTMS, elle est utilisée dans le traitement de plusieurs troubles psychiatriques et neurologiques. Cependant ses mécanismes d'action restent mal connues, ainsi que l'influence de différents paramètres de stimulation, tels que l'intensité et la fréquence, sur les résultats obtenus.

Cette thèse étudie la rTMS dans des modèles animaux de deux maladies neurologiques. Dans la première, la dépression, la rTMS est un traitement autorisé, alors que pour la deuxième, la maladie d'Alzheimer, la rTMS reste une nouvelle technique expérimentale. Ces deux maladies ont été choisies pour leurs pathologies différentes, afin de pouvoir étudier la possibilité d'optimiser les paramètres de rTMS pour traiter les aspects particuliers de ces maladies.

Nous avons d'abord évalué les effets de la rTMS dans un modèle murin de la dépression en faisant varier l'intensité de la stimulation. Les souris ont reçu une lésion du bulbe olfactif (abréviation de l'anglais: OB) puis ont été traitées avec la rTMS à basse intensité (low intensité: LI-rTMS; 12mT), moyenne intensité (MI-rTMS, 90mT) ou haute intensité (HI-rTMS, 1.2T) appliquée à une fréquence constante de 10Hz, 5 jours par semaine pendant 4 semaines, et ciblée sur le cortex préfrontal. Le modèle OB a induit une hyperactivité psychomotrice dans de test de la nage forcée, phénotype qu'a atténuée la MI-rTMS et l'HI-rTMS. Cependant, seule la MI-rTMS a provoquée une augmentation du BDNF (brain-derived neurotrophic factor) et de la neurogénèse dans des régions spécifiques du cerveau. De plus, HI-rTMS a normalise la concentration plasmatique de l'acide α -amino-n-butryrique et de la 3-methylhistidine. L'analyse des différentes voies métaboliques à l'aide du logiciel Ingenuity Pathway Analysis a démontré des variations significatives dans le métabolisme de la glutamine, ainsi que la signalisation au glutamate dans le modèle OB lui-même, ainsi qu'à la suite des traitements MI-rTMS and HI-rTMS.

Nous avons ensuite étudié les effets de la LI-rTMS en faisant varier la fréquence dans les souris PS1M146V, un modèle transgénique de la maladie d'Alzheimer. Des souris femelles PS1M146V et C57BL6/J âgées de 6 et 12 mois ont été stimulées 10 minutes par

jour pendant 14 jours, ciblant l'hippocampe. Nous avons comparé 4 fréquences différentes de LI-rTMS : deux fréquences excitatrices (BHFS et 10Hz) et deux fréquences inhibitrices (1Hz and cTBS). Dans les souris âgées de 6 mois, les deux fréquences excitatrices ont amélioré les déficits de mémoire spatiale dans la piscine de Morris et ont réparé les épines dendritiques anormaux. Cependant, les deux fréquences inhibitrices ont été moins efficaces: 1Hz stimulation a induit une amélioration partielle et cTBS a été sans effet, ou pour certaines mesures, a provoqué des effets néfastes. Aucune fréquence n'a augmenté la neurogénèse chez les souris PS1M146V, mais l'expression de la BDNF a été légèrement augmentée par la stimulation à 10Hz. Le manque d'effet de la LI-rTMS sur le comportement dans le labyrinthe en croix surélevé suggère que l'amélioration de la mémoire spatiale dans ces souris n'est pas suite à une réduction d'anxiété. Dans les souris plus âgées (12 mois), la stimulation a également amélioré la mémoire spatiale et la morphologie des épines dendritiques des souris PS1M146V. En revanche, chez les souris témoins (C57BL6/J) la stimulation n'a eu d'effet qu'une réduction de la mémoire spatiale.

Les résultats de ces deux études suggèrent qu'il est possible de formuler des traitements spécifiques aux différentes maladies neurologiques et psychiatriques, en faisant varier l'intensité, la fréquence, et éventuellement la région du cerveau ciblée par le traitement. Pour une pathologie telle que la dépression, qui implique une diminution d'activité dans les circuits limbiques, une rTMS à intensité plus forte dirigée sur le cortex frontal semble la plus efficace. Cependant, dans la maladie de l'Alzheimer, où le maintien des synapses et de leur bon fonctionnement est affaibli, la rTMS à basse intensité (LI-rTMS), qui pourrait moduler des mécanismes cellulaires afin de renforcer les synapses, semble mieux adaptée.

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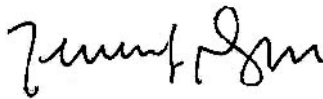
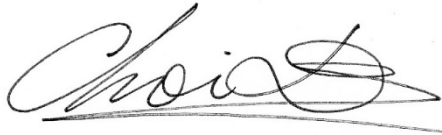

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Authorship Declaration: Co-Authored Publications

This thesis contains work that has been published and prepared for publication.

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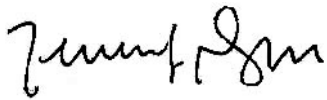
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Abbreviations

5HT	5-hydroxytryptamine
AABA	α -amino-n-butyric acid
ACTH	adrenocorticotropic hormone
AD (s/f)	Alzheimer's disease (sporadic/ familial)
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APP	amyloid precursor protein
A β	β -amyloid
BDNF	brain derived neurotrophic factor
BHFS	biomimetic high frequency stimulation
BSA	bovine serum albumin
Ca ²⁺	calcium
CAMKII	calmodulin dependent kinase II
Creb	cAMP response element binding
DLPFC	dorsolateral prefrontal cortex
EdU	5-ethynyl-2'-deoxyuridine
ELF-EMF	extremely low frequency electromagnetic fields
ELISA	enzyme-linked immunosorbent assay
EPSC	excitatory post synaptic current
ER	endoplasmic reticulum
FDA	Food & Drug Administration
FRET	Fluorescence Resonance Energy Transfer
FST	forced swim test
GABA	gamma-Aminobutyric acid
HI	high intensity
HPA	hypothalamic-pituitary-adrenal
Hz	hertz
IP3R	inositol triphosphate receptor
IPA	Ingenuity Pathway Analysis
LCMS	liquid chromatography–mass spectrometry
LI	low intensity
LTD/P	long term depression/potential

MCI	mild cognitive impairment
MEP	motor evoked potentials
MI	medium intensity
mm	millimetres
msec	milliseconds
MSO	maximum stimulator output
MT (r)	motor threshold (resting)
MWM	Morris water maze
NFTs	neurofibrillary tangles
NMDA	n-methyl-d-aspartate
OB	olfactory bulbectomy
PBS (T)	phosphate buffer solution (triton)
PCR (qRT)	polymerase chain reaction (quantitative real time)
PS1/2	presenilin 1/2
PSD95	post synaptic density 95
RNA (m)	ribonucleic acid (messenger)
rTMS	repetitive transcranial magnetic stimulation
RyR	ryanodine receptor
SGZ	subgranular zone
SSRI	selective serotonin reuptake inhibitor
STIM2	Stromal interaction molecule 2
SVZ	subventricular zone
T(m)	Tesla (mili)
TBS (i/c)	theta burst stimulation (intermittent/continuous)
tDCS	transcranial direct current stimulation
TMS	transcranial magnetic stimulation
TRD	treatment resistant depression
VGCC	voltage gated calcium channels

Chapter 1:

Introduction

In my thesis, I have used preclinical animal models of human neurological conditions to try to understand how repetitive transcranial magnetic stimulation (rTMS) affects the brain. In this introduction, I will begin by reviewing what is known about neuromodulation and this particular method, rTMS, and the mechanisms of action that have been identified in humans and animal models. My focus will be on how altering different parameters of the stimulation affects the outcome, especially lower intensity forms of rTMS. I will discuss the therapeutic use of rTMS to treat the two neurological conditions that are the focus of my research (depression and Alzheimer's disease). In addition, I will review the current literature and understanding of these neurological conditions. Finally, I will review the animal models that have been used to explore the biological basis of these conditions and their treatment. Overall the aim of the literature review is to highlight what is known about rTMS and how it can be used therapeutically, and how preclinical models can contribute to better understand disease states and treatments for human health.

1. Neuromodulation

Once considered a static system, it is now well known that the central nervous system undergoes changes to its structure and function, known as neuroplasticity. These changes are essential for learning and memory, but can also contribute to the addition of new behaviours such as addiction (Bailey et al. 2015). After a neurological insult this plasticity is also important for recovery, most evidently after a stroke or traumatic brain injury (Munoz-Cespedes et al. 2005; Ward 2011). However, recovery from plasticity is limited, so considerable research is undertaken to externally promote and modulate this neuroplasticity. Drugs or other interventions such as exercise can initiate neuroplasticity or metaplasticity; which is defined as changes that affect subsequent neuroplasticity occurring (Voss et al. 2017). Another method to induce neuroplasticity, and the focus of this thesis, is non-invasive brain stimulation.

Non-invasive brain stimulation methods have been used to stimulate the brain for over 150 years (Muller-Dahlhaus & Vlachos 2013). Over time, advancements in technology have led to better stimulation methods that can target specific brain regions and are relatively safe for treatment of patients (Loo et al. 2008). Stimulation can either be

applied using electrical currents, such as with transcranial electric stimulation, electroconvulsive therapy or transcranial direct current stimulation, or using electromagnetic induction such as with transcranial magnetic stimulation (Wagner et al. 2007).

1.1 Transcranial Magnetic Stimulation

Transcranial magnetic stimulation (TMS) provides a way of non-invasively stimulating the brain in non-anesthetized human subjects. First used by Barker et al. (1985), this form of stimulation applies Faraday's theory of electromagnetic induction to induce a stimulatory pulse. When a strong electric current is passed through a wire coil a perpendicular magnetic field is generated. When this magnetic field passes through a conductor, an electric current is produced (Figure 1.1) (Muller-Dahlhaus & Vlachos 2013). In the case of TMS, brain tissue acts as the conductor as neurons maintain electrical gradients that can deduce this signal (Huerta & Volpe 2009).

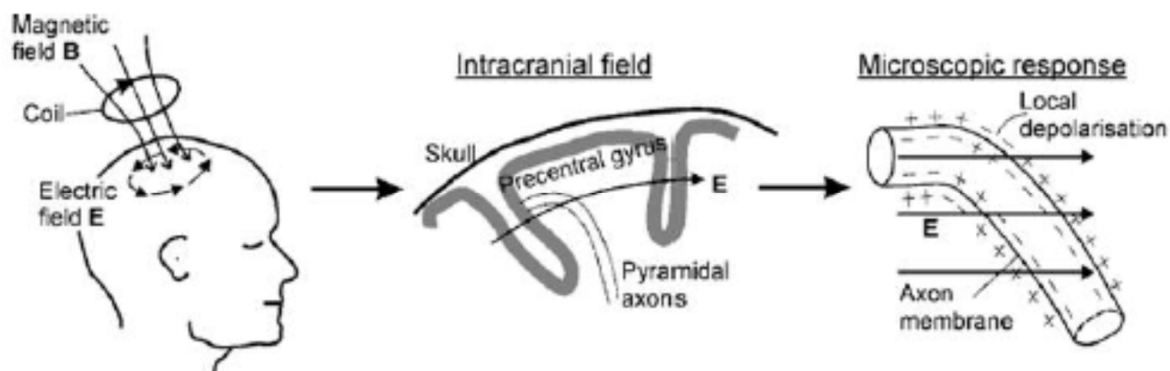


Figure 1.1 Representation of TMS induced electrical currents in the brain. The magnetic field produced from the rTMS coil causes an underlying electrical field in the brain. This field stimulates various axons in the underlying cortex causing depolarization and signalling to occur (Ruohonen 1998).

Axons are the primary target of TMS. The induced current causes an influx of ions which results in a change in membrane potential that causes depolarisation and initiates an action potential (Banerjee et al. 2017; Huerta & Volpe 2009). The process is nonselective but various factors such as the length or bending of the axon, the level of myelination, or cell type can affect depolarization (Ruohonen & Ilmoniemi 1999; Siebner et al. 2009). Magnetic fields have also been shown to affect apical dendrites of pyramidal cells, and therefore these could also be considered as a target of stimulation if they are oriented along the induced electric field (Lee & Fried 2017). As well as the type of the neuron, the characteristics of the stimulation affect the response to TMS. The amplitude, waveform

and duration of the pulse and, the diameter and shape of the coil all can affect the stimulation (Pell et al. 2011).

The most visible outputs of this initiation of action potentials are motor evoked responses to a single pulse of TMS focused over the motor cortex (Huerta & Volpe 2009; Pell, Roth & Zangen 2011). But in all areas of the brain TMS can cause the acute release of excitatory, inhibitory or modulatory neurotransmitters depending on the neuron population targeted (Huerta & Volpe 2009). The effects of the release of these neurotransmitters are acute, however when trains of these TMS pulses are put together known as repetitive transcranial magnetic stimulation (rTMS) more long-lasting effects are seen.

1.2 Repetitive Transcranial Magnetic Stimulation

Repeatedly depolarising neurons using rTMS can induce long lasting neuroplastic effects. This has been demonstrated by the use of different rTMS protocols which cause long term potentiation (LTP)- or long term depression (LTD)-like effects (Hallett 2007). Whether LTP or LTD, or another metaplastic effect occurs after rTMS is dependent on the parameters of the stimulation. These include, but are not limited to, the frequency, which is the number of pulses per second, the coil shape, which can determine the focality, and the intensity of the stimulation.

1.2.1 Frequency

Frequencies can be classified simply according to whether they induce excitatory or inhibitory changes (Figure 1.2). In general, low frequencies less than 1Hz have LTD-like effects and high frequencies above 5Hz have LTP-like effects (Hallett 2007). These LTP and LTD-like changes can be further strengthened with complex frequencies. Complex frequencies are often developed to replicate endogenous firing patterns within the brain or nerves. Theta burst stimulation (TBS) was developed to mimic the theta firing patterning in the brain. The frequency involves three pulses at 50Hz repeated at 5Hz either continuously (cTBS) or for a 10 burst train with an inter train interval of 8 seconds (iTBS) (Huang et al. 2005). The effects of cTBS and iTBS are opposing, with cTBS causing inhibition and iTBS, excitation (Huang et al. 2011). Another complex frequency, Biomimetic High Frequency (BHFS), was developed to replicate endogenous firing patterns around nerves during exercise. This frequency consists of short 50msec bursts repeated at 6.67Hz for the first minute, 10.01Hz for the next eight minutes and 8.75Hz

for the final minute (Rodger et al. 2012). Although the effects of BHFS have never been investigated using high intensity stimulation in human, evidence from mouse *in vivo* studies suggest it has excitation-like effects (Seewoo et al. 2018).

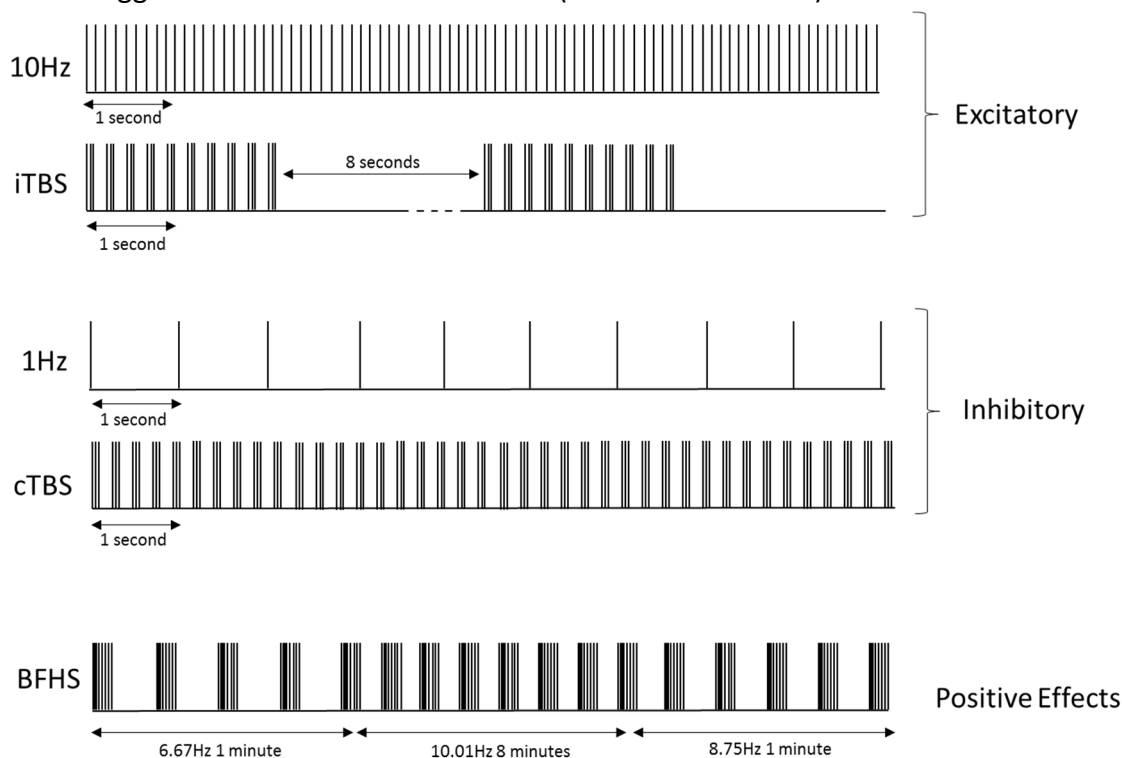


Figure 1.2 Both simple and complex rTMS frequencies can induce specific effects. The pattern of rTMS stimulation can result either excitatory or inhibitory effects. Two frequencies that are known to induce excitation are 10Hz and iTBS (three pulses at 50hz repeated at 5Hz for a 10 pulse train with an intertrain interval of 8 seconds) and two frequencies known to cause inhibition are 1Hz and cTBS (three pulses at 50Hz repeated continuously). Biomimetic High frequency (BHFS) is another complex frequency and though its exact effect on excitation/inhibition is unknown it has positive effects in animal models of dysfunction.

1.2.2 Coil Shape

The shape of the coil influences the focality and strength of the stimulation. Common coil shapes have a simple circle, a figure-of-8 butterfly or an H-coil design (Hallett 2007). The electric field produced under a figure-of-8 coil has greater focality and strength in comparison to a circular coil, but there is always a rapid drop off in intensity the further away from the coil the targeted area is (Deng et al. 2013). Deeper stimulation could be achieved with these coils by increasing the intensity of the stimulation, however this would result in unsafe stimulation levels of superficial targets (Deng et al. 2014). Instead other designs, such as the H-coil, can be used to reach deep brain structures but with a reduced focality (Deng, Lisanby & Peterchev 2014). It is therefore important to be critical and analyse the need of focality or depth before choosing a specific coil design.

1.2.3 Intensity

The intensity of the stimulatory pulse will also affect rTMS. A threshold level of stimulation is needed to induce action potentials in neurons (Huerta & Volpe 2009). In humans this is often measured as the motor threshold (MT) which is the lowest intensity of stimulation needed to induce a measurable motor output (Herbsman et al. 2009). This is variable between people as the skull-to-cortex distance changes with population and age (Herbsman et al. 2009; Rossini et al. 2015). Lower intensity forms of rTMS do not result in these motor outputs however, they have been shown to effect other processes in the brain (Rodger et al. 2012; Tang et al. 2016a).

1.3 Effects of High-Intensity rTMS

In addition to measurable motor outputs, there are many biological changes induced by high intensity rTMS. These include changes to plasticity, the regulation of various neurotransmitter and hormonal systems, and influences to neurogenesis.

1.3.1 Synaptic Plasticity

The influence rTMS can have on plasticity has been consistently demonstrated and as previously mentioned, different frequencies of rTMS affect cortical excitability. These changes have been proposed to be resultant of changes to synaptic plasticity (Hallett 2007). Synapses, which are the point at which one neuron makes a functional connection to another, change their function in response to inputs. The two main ways a synapse can undergo plasticity is by potentiation or depression of the future synaptic responses, or adapting the structural component of the synapse (Citri & Malenka 2008; Yang et al. 2008). Various *in vitro* and *in vivo* studies have examined the effects of rTMS at synaptic and molecular levels to attempt to explain how rTMS influences already existing plasticity mechanisms at the synapse.

1.3.1.1 Long Term Potentiation

At a single synapse there are distinct mechanisms for LTP or LTD (Granger & Nicoll 2014). Generally, it is excitatory synapses which contain glutamatergic n-methyl-d-aspartate (NMDA) receptors that are susceptible to LTP (Citri & Malenka 2008). At these synapses glutamate binds to NMDA and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors which induces a post-synaptic response. In normal brain activity glutamate is released in a temporal pattern depending on the incoming action potential firing rate and the size and frequency of the pre-synaptic inputs results in LTP or LTD

occurring (Citri & Malenka 2008). We note that NMDA-independent mechanisms of synaptic plasticity occur in many brain regions (Kato et al. 2009). However, this thesis has chosen to focus primarily on NMDA-dependent plasticity, especially at glutamatergic synapses, because it is the best characterised mechanism in rTMS effects. In addition, abnormalities in this type of plasticity are directly relevant to neurological disorders such as depression and AD.

Long term potentiation can be measured directly using excitatory post synaptic currents and potentials (EPSCs) *in vitro* and *in vivo*. This was first demonstrated by Bliss and Lomo (1973), where a high frequency stimulation resulted in increased responses to further stimulation in hippocampal neurons. Depending on the input these facilitated responses can last from minutes to hours *in vitro*, to days *in vivo* (Citri & Malenka 2008). Measured directly after rTMS, long lasting LTP-like electrophysiological changes have also been recorded *in vitro* (Lenz et al. 2015; Tokay et al. 2009; Vlachos et al. 2012). Longer term stimulations can also effect spontaneous mini-EPSCs (Tan et al. 2013b), which are modulated by the length (number of days) and intensity of treatment (Ogiue-Ikeda et al. 2003). Although this LTP-like response of synapses can be easily measured after high frequency rTMS, the mechanism by which it occurs is still being studied.

rTMS has been shown to affect various aspects of LTP induction (Lenz et al. 2016; Lenz et al. 2015; Tokay et al. 2009; Vlachos et al. 2012). Under normal conditions, induction of LTP is dependent on NMDA and highly dynamic AMPA receptors. In response to an LTP inducing stimulus, new AMPA receptors are transported to the membrane and existing receptors are phosphorylated, events which together enhance transmission therefore increasing the response of the postsynaptic membrane to incoming stimuli (Lüscher & Malenka 2012). rTMS directly affects this mechanism, increasing AMPA receptor density which is NMDA receptor dependent (Lenz et al. 2016; Lenz et al. 2015; Tokay et al. 2009; Vlachos et al. 2012). Other regulators of membrane potential have also been shown to be affected by rTMS including sodium, potassium and voltage gated calcium channels (VGCCs) (Tan et al. 2013b). Additionally, if there is a very large LTP inducing stimulus this is known to cause protein and neurotrophin translation, and changes to gene expression, which contributes to a stable and longer lasting LTP response (Raymond 2007). One neurotrophin of interest that is upregulated after LTP is brain derived neurotrophic factor (BDNF) and rTMS has been shown to increase BDNF

after stimulation (Gersner et al. 2011; Muller et al. 2000; Wang et al. 2011). BDNF is involved in many neuronal processes, in particular the regulation of synaptic scaffolding proteins which results in changes to dendritic spine morphology (Hu et al. 2011; Yoshii & Constantine-Paton 2007).

1.3.1.2 Long Term Depression

In contrast to the wealth of knowledge and research in relation to the mechanisms of rTMS induced LTP-like changes, the mechanisms of LTD-like effects after low frequency stimulation are less understood. No studies have directly shown a decrease in EPSCs after low frequency stimulation, however one study which examined inhibition of motor evoked potential amplitude following 1Hz stimulation in a rodent model showed this effect was dependent on NMDA receptors (Muller et al. 2014), consistent with an NMDA-dependent LTD-like mechanism reviewed here.

1.3.1.3 Spine Morphology

Another component of synaptic plasticity on which rTMS has an effect is dendritic spine morphology. Dendritic spines are small protrusions along the dendrite onto which synapses occur. These spines have distinct shapes which are related to their functional significance (Hering & Sheng 2001). The most functionally important shape is the mushroom-shaped spine, these have a bulbous head with a thin neck, this shape confines signalling mechanisms to the spine and creates defined microdomains along the dendrite (Bosch et al. 2014). An important feature of these spines is that they can undergo morphological changes. For example, the growing of a spine is activity dependent and coincides with LTP induction (Kopec et al. 2006; Yang et al. 2008). The result being these large, mushroom shaped spines that can accommodate more AMPA receptors and consist of increased structural proteins creating stability and longer LTP (Matsuzaki et al. 2001; Yang et al. 2008). In contrast, an LTD inducing stimulation causes the shrinkage of spines and in extreme cases blocking of all neuronal activity changes the spines into long immature protrusions that lack a spine head (Verpelli et al. 2010; Zhou et al. 2004). rTMS has been shown to induce structural changes in dendritic spine size, but not number, consistent with its effect in strengthening already made connections not inducing new ones (Vlachos et al. 2012).

1.3.1.4 Calcium involvement in Synaptic Plasticity

It has been shown that high intensity rTMS increases calcium (Ca^{2+}) within neurons (Banerjee et al. 2017). Calcium plays an important messaging role in the initiation and regulation of LTP and LTD (Brini et al. 2014), therefore the previously discussed effects of the stimulation could be attributed to its effect on this ion. Calcium signals differ in size and purpose depending on their source and location in the neuron (Goldberg et al. 2003). Post-synaptic calcium enters through VGCCs, NMDA and AMPA receptors. Internally, calcium release from the endoplasmic reticulum (ER) also plays an important role in the initiation and maintenance of LTP and LTD (Brini et al. 2014). Its mechanism of action is through the activation of various proteins which can result in receptor activation and trafficking, transcriptional regulation, and intracellular structural changes (Brini et al. 2014). Two proteins of specific interest because of their relationship to LTP and LTD are calmodulin dependent kinase II (CAMKII) and calcineurin.

1.3.1.5 CAMKII and Calcineurin

One specific way calcium can regulate LTP and LTD is through activation of calmodulin dependent kinases and phosphatases. When calcium binds to the fast buffer, calmodulin (Faas et al. 2011) it can activate specific proteins. Two of these major synaptic proteins are CAMKII and calcineurin (also known as protein phosphatase 2B). Specifically, in the cortex and hippocampus, CAMKII is thought to contribute to LTP (Giese et al. 1998) and calcineurin to LTD (Zeng et al. 2001).

CAMKII

CAMKII has an important role in the development of LTP. Knockdown and inhibition studies show LTP is perturbed and there is impaired learning and memory in animals with altered CaMKII function (Giese et al. 1998; Murakoshi et al. 2017; Rossetti et al. 2017). Though its primary form of activation is through binding of Ca^{2+} /calmodulin complexes it can stay in an active phosphorylated state if two adjacent subunits are bound (Bossuyt & Bers 2013). CAMKII can become activated from calcium entering through NMDA receptors or being released from ER stores (Lu et al. 2014).

Activated CAMKII has spatially specific roles, this allows for spine specific LTP changes. It binds to the NMDA subunit NR2B, which is necessary to induce LTP (Strack & Colbran 1998), contributes to the trafficking and conductance of AMPA receptors (Appleby et al. 2011; Kristensen et al. 2011; Opazo et al. 2010) and responds to VGCC calcium influxes

by transporting calmodulin to the nucleus to activate cAMP response element binding (CREB) (Ma et al. 2014; Wheeler et al. 2008; Yan et al. 2016). It is also involved in maintaining LTP due to its interaction with NMDA (Barcomb et al. 2016; Rossetti et al. 2017; Sanhueza et al. 2011; Yamagata et al. 2009).

Calcineurin

Calcineurin involvement at the synapse is related to LTD and the suppression of synaptic activity. When calcineurin is suppressed LTD is inhibited (Mulkey et al. 1994; Zeng et al. 2001) and LTP and short term memory are enhanced (Ikegami et al. 1996; Malleret et al. 2011; Zeng et al. 2001). Conversely, when calcineurin expression is enhanced, LTP is diminished (Winder et al. 1998). Interestingly, it also plays a role in suppressing inhibitory circuits when LTP is induced in excitatory circuits (Lu et al. 2000)

When activated, calcineurin can directly affect the functions of intracellular proteins and can have indirect influences through modulation of downstream signalling cascades. For mechanisms directly related to LTD, activated calcineurin can modulate the phosphorylation state of AMPA receptors and cause endocytosis (Beattie et al. 2000; Tavalin et al. 2002). It also affects the expression of potassium voltage gated channels which regulate the excitability of the post synaptic membrane (Lin et al. 2011). These mechanisms lead to the initiation of LTD or diminish LTP.

CAMKII or Calcineurin: Calcium Signals?

Research has investigated how either CAMKII or calcineurin can be selectively activated, when they are both activated by the same Ca²⁺/Calmodulin complexes and are both present at similar levels in the brain (Sola et al. 1999). It was first suggested that a large increase in post-synaptic calcium selectively activates CAMKII, while a moderate rise activates calcineurin (Lisman 1989). However, several studies indicate that this hypothesis is too simplistic (Yang et al. 1999). Because dendritic calcium buffering is extremely fast, local changes in calcium concentration occur as a series of pulses that parallel temporal spiking patterns, rather than a steady gradual influx (Cornelisse et al. 2007). This means that decoding calcium entry into either activating CAMKII or calcineurin may be more dependent on the frequency of calcium influx rather than the overall amount (Li et al. 2012). This was elegantly proven in a study by Fujii et al. (2013) which showed the differential activation of either CAMKII or calcineurin after different frequencies and train lengths of glutamate uncaging at single synapses (Figure 1.3).

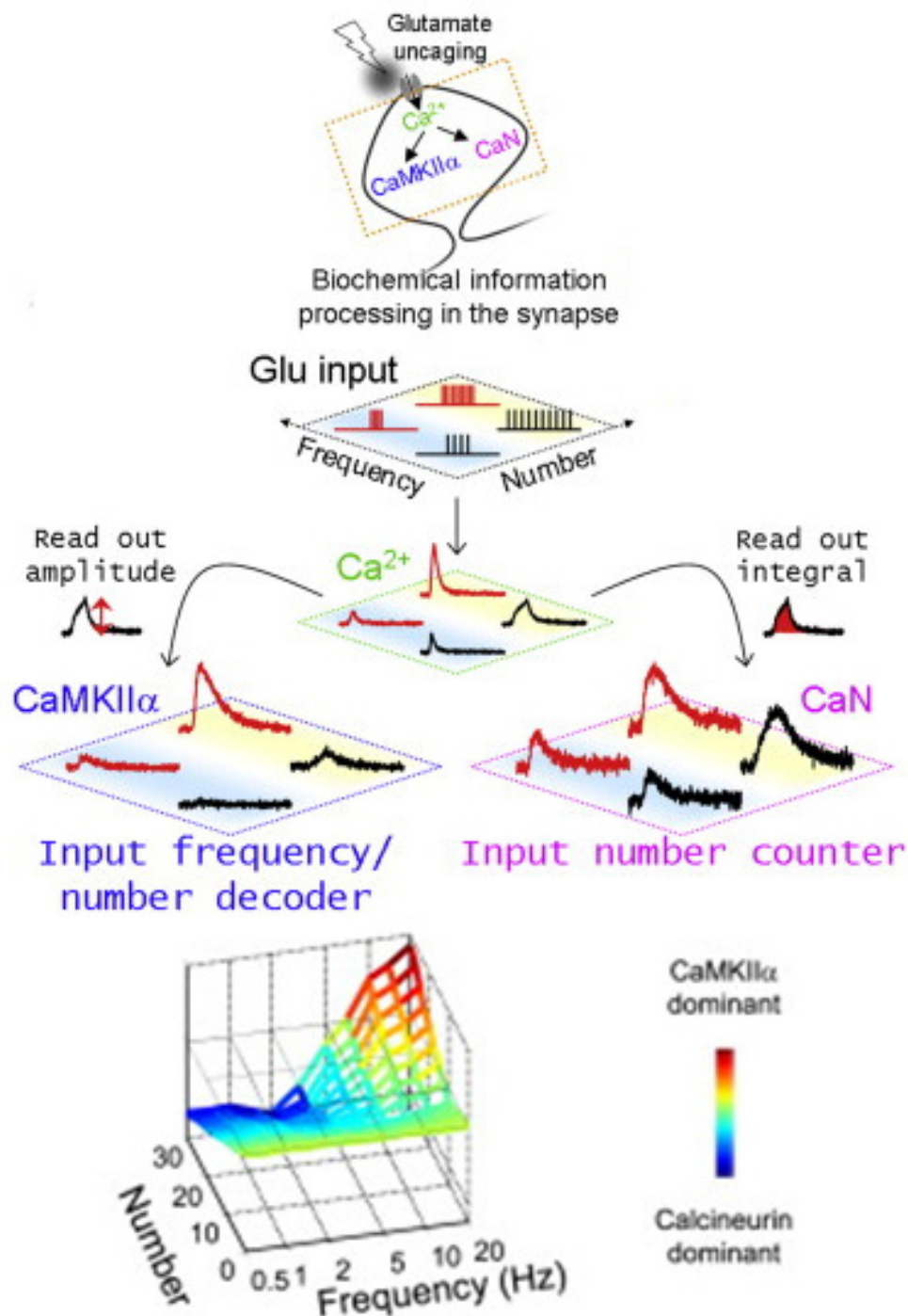


Figure 1.3 Activation of either CAMKII or calcineurin depends on both frequency and number of inputs.

A summary of a study by Fujii et al. (2013) which investigated the frequency specific effects on the post synaptic response, they used FRET imaging to visualise the activation of either CAMKII or calcineurin after specific frequency and number of glutamate uncaging inputs at an individual synapse. It was found that calcineurin was responsive to most stimulation protocols and increased activation on the input number, when a higher frequency longer input occurred there was shift to more CAMKII activation, though calcineurin was still activated just at a less amount than CAMKII.

The frequency dependent activation of CAMKII and calcineurin during normal brain activity suggests that these proteins could also function to decode rTMS signals into LTP or LTD. Limited research has examined the direct activation of CAMKII or calcineurin after rTMS: Lenz et al. (2016) showed that calcineurin was involved in the LTD response

of neurons to low frequency stimulation, and upregulation of CAMKII has been shown after high frequency rTMS *in vitro* (Baek et al. 2018). These results confirm that rTMS can influence CAMKII or calcineurin depending on the frequency delivered and therefore this mechanism may be highly involved in translating different frequencies of rTMS to either LTP or LTD.

1.3.1.6 Inhibitory Plasticity

The studies described above have focused solely on the effects of rTMS on excitatory glutamatergic circuitry, which have been shown to be highly responsive to rTMS (Tokay et al. 2009; Vlachos et al. 2012). However, other neuronal cells such as cortical GABAergic interneurons, also have clear responses to stimulation (Lenz & Vlachos 2016). Mounting evidence has shown that rTMS can both increase and decrease activity from GABAergic interneurons in the cortex, and this effect is frequency dependent (Labeledi et al. 2014; Trippe et al. 2009; Volz et al. 2013). Therefore, depending on the stimulation parameters, rTMS can activate a separate mechanism by which it can induce plasticity, modulating inhibitory interneurons. This modulation of GABAergic neuron activity has generated the “disinhibition” hypothesis of rTMS. It has been hypothesised that the LTP-like effects of rTMS are not in fact due to a direct induction from rTMS, but due to a depression of inhibitory synapses, which would have previously reduced a potentiated response (Lenz et al. 2016).

1.3.2 Influence on Regulatory Molecules

1.3.2.1 Glutamate and GABA

Glutamatergic and GABAergic systems in the brain are related directly to excitatory and inhibitory neurons respectively and therefore understanding the effect of rTMS on the concentrations of these two neurotransmitters may indicate its effect on the interplay of their circuitry. In humans, direct imaging of glutamate and gamma-Aminobutyric acid (GABA) levels in tissue has been made possible using proton magnetic resonance spectroscopy. Single and multiple rTMS sessions can increase glutamate and GABA levels in the cortex of healthy volunteers (Michael et al. 2003) and improves those with deficits in GABAergic signalling (Baeken et al. 2017; Dubin et al. 2016). Increases in glutamate and GABA content in the hippocampus and striatum but not the midbrain and hypothalamus have also been found in rats following rTMS (Yue et al. 2009). As the

mechanisms of rTMS rely on both excitation and inhibition as previously described, it is no surprise that glutamate and GABA are affected by the stimulation.

1.3.2.2 Monoamines

There is no clear consensus for the impact of rTMS on monoamine levels, with acute and chronic rTMS showing different effects. Acute rTMS transiently affects monoamine concentrations (Ben-Shachar et al. 1997; Löffler et al. 2012) and amino acid levels (Keck et al. 2000), while chronic rTMS has little effect on noradrenaline, dopamine, serotonin or their metabolites (Ben-Shachar et al. 1999). Chronic rTMS however exhibits an effect on the structural elements of neurotransmitter function. Following chronic high frequency rTMS in mice several changes to the monoamine system have been demonstrated: including reduced serotonin and increased noradrenaline transporter mRNA, and increased uptake and binding of noradrenaline (Ikeda et al. 2005), as well as reduced sensitivity of hypothalamic serotonin receptors (Gur et al. 2004). This demonstrates the ability for rTMS to modulate neurotransmitter, especially monoamine, systems in the brain.

1.3.2.3 Neuroendocrine

In addition to changes in neurotransmitters, other regulatory molecules are changed by rTMS, including hormones. Changes to the neuroendocrine system have been examined following rTMS in both humans and animals with similar results being found across species. In humans rTMS causes a decrease in thyroid stimulating hormone and cortisol levels immediately following treatment (Cohrs et al. 2001; Evers et al. 2001). A similar immediate effect of rTMS in rodents has yet to be observed for corticosterone (cortisol equivalent) (Gur et al. 2004), however a decrease in another hormone important in the stress response, vasopressin, has been shown immediately after rTMS stimulation in rodent models (Keck et al. 2000). Although the acute effects of rTMS are interesting, chronic stimulation alters the regulation of hormones in both humans and animals. After chronic stimulation in humans there is a decrease in the response to dexamethasone, used to indicate hyperactivation of the stress hormone system (Zwanzger et al. 2003), and in rodents rTMS stimulation reduced both the corticosterone and adrenocorticotrophic hormone (ACTH) response to a stressor (Czéh et al. 2002; Hedges et al. 2003; Hedges et al. 2002).

1.3.3 Neurogenesis

rTMS has also been shown to influence another phenomenon which is involved in neuroplasticity, neurogenesis. Adult neurogenesis is the formation of new neurons after development (Ming & Song 2011) and was first observed in the rat dentate gyrus (Altman & Das 1965). This observation was then extended to humans looking at 14-Carbon levels or 5-bromo-2'-deoxyuridine (BrdU) staining in the brain (Ernst & Friséen 2015) although these findings remain controversial (Sorrells et al. 2018). Neurogenesis occurs in two main locations in the brain: the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus in the hippocampus. A proportion of the newly born neurons integrate into existing circuits and become functional. Specifically in the SGZ, neurons migrate to the granule layer of the dentate gyrus and project their growing axons to the CA3 and dendrites into the molecular layer (Ming & Song 2011; Zhao et al. 2006). Each of these stages is governed by a variety of environmental, physiological and pharmacological stimuli, which determine the survival and differentiation of these neurons (Ming & Song 2011). Neuronal activity, particularly GABAergic and glutamatergic inputs, affect the proliferation, integration and fate determination of these immature neurons (Espósito et al. 2005; Ge et al. 2005; Song et al. 2012; Tashiro et al. 2006). Other neurotransmitters, such as serotonin, norepinephrine and dopamine, as well as other transcription factors are important in differentiation and survival (Ming & Song 2011).

There are currently only a few studies that examine the effect of rTMS on neurogenesis but all concur that it can promote this function. Following chronic rTMS, rats had an increase in newly proliferated neurons (Ueyama et al. 2011). Also, in animal models with decreased neurogenesis, proliferation was increased following rTMS (Czéh et al. 2002; Feng et al. 2012). However, the long term survival of these proliferated cells was decreased, suggesting that they did not integrate into functional networks (Czéh et al. 2002). Proliferation was also examined in the SVZ after different frequencies of rTMS, with the finding that the duration of stimulation was more important than the frequency for increasing proliferation of neurons (Abbasnia et al. 2015).

Although rTMS has been shown to affect neurogenesis, the factors that regulate rTMS-induced neurogenesis have yet to be determined. Potentially, increases in excitatory activity, which is known to positively influence neurogenesis or the increase in certain

growth factors such as BDNF which is known to be influenced by rTMS (Czéh et al. 2002; Gersner et al. 2011) could be involved.

1.3.4 Effects of Low Intensity rTMS

Low intensity rTMS (LI-rTMS), which will be explored in this thesis, can also be used to produce unique effects making it an interesting alternative to HI-rTMS. As LI-rTMS does not generate the same depolarisation dependent mechanisms as HI-rTMS, but still induces plasticity (Rodger et al. 2012), it is important to understand these different mechanisms of action. Recent research investigating LI-rTMS with *in vitro* and *in vivo* models has begun to elucidate some of the mechanisms. Acute LI-rTMS stimulation released calcium from ER stores in neurons (Grehl et al. 2015) and increased BDNF in specific brain areas (Makowiecki et al. 2014). It is likely that this calcium rise could affect various neuronal processes (Figure 1.4), however these have yet to be explored experimentally. In addition, chronic LI-rTMS stimulation has been shown to improve abnormal connections within the brain without affecting normal brain function (Makowiecki et al. 2014; Poh et al. 2018; Rodger et al. 2012).

Other forms of low intensity stimulation that do not depolarise neurons could share similar mechanisms with LI-rTMS. Transcranial direct current stimulation (tDCS) and extremely low frequency electromagnetic fields (ELF-EMF) are both low intensity forms of stimulation but they have profound effects on the brain. Rather than activating neurons directly to fire, they may cause changes in the capacity for plasticity; metaplasticity.

Transcranial direct current stimulation (tDCS), which involves the application of weak electrical currents to the brain increases cortical excitability (Nitsche & Paulus 2001). Depending on the direction of the current it is hypothesised to cause neurons to hyper- or hypo-polarise affecting their excitability (Stagg & Nitsche 2011). TDCS has already been trialled as a treatment for various neuropsychiatric disorders and as a neurorehabilitation tool (Sánchez-Kuhn et al. 2017; Zhao et al. 2017a). However, one of the limitations of tDCS is the lack of focality, as the current must pass between two electrodes which are generally placed on either side of the head (Woods et al. 2016).

Extremely low frequency electromagnetic fields are the signals produced by electrical appliances and their effect on the brain has also been studied. The intensity of these

stimulations is comparable to LI-rTMS (<20mT) but the delivery method lacks focality and stimulations use very high frequency (>30Hz) (Guerriero & Ricevuti 2016). This type of stimulation has a clinical effect in altering pain thresholds (Choleris et al. 2002; Ghione et al. 2005). Interestingly, research has shown a similar ELF-EMF may have a similar mechanism of action to LI-rTMS as it increases calcium within both neuronal (Li et al. 2014b; Luo et al. 2014) and non-neuronal cells (Carson et al. 1990).

In some cases a non-focal stimulation is appropriate for the type of treatment needed. But as targeted treatment with tDCS or ELF-EMF is not feasible, LI-rTMS can be considered an appealing alternative as it has been shown to have promising effects altering brain plasticity in a region dependent manner (Makowiecki et al. 2014).

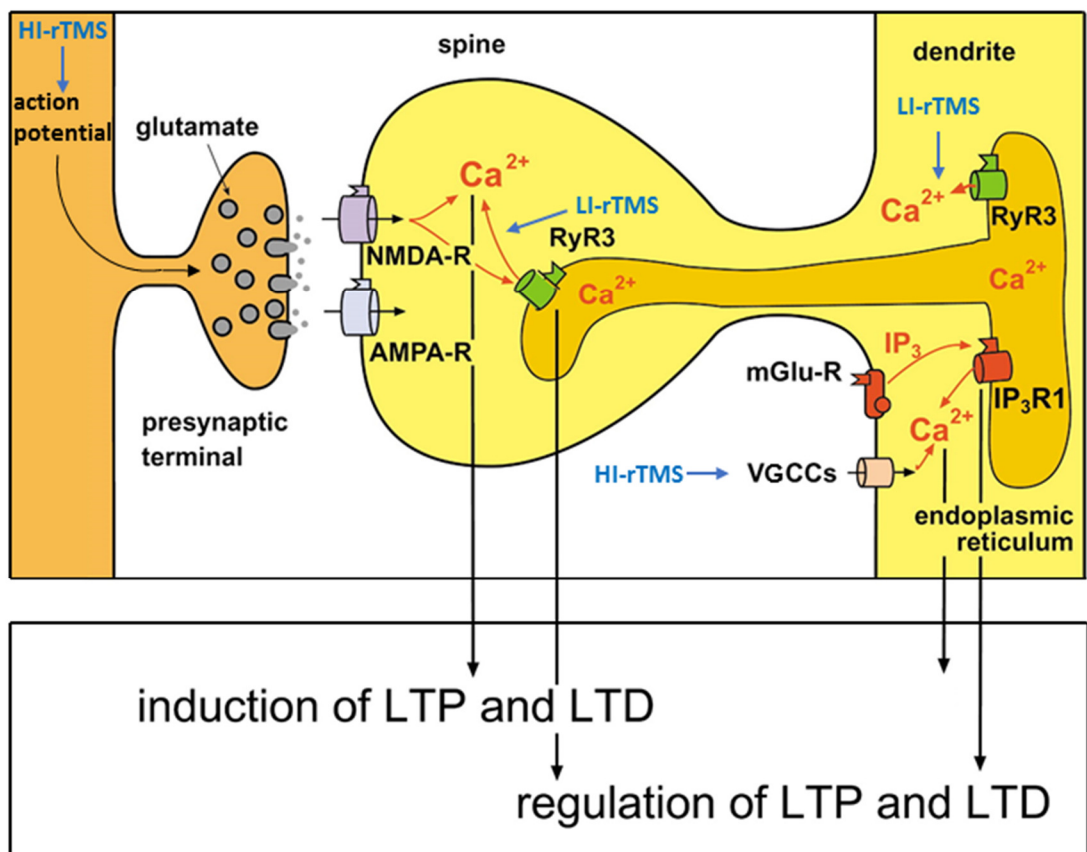


Figure 1.4 Potential effects of HI- and LI-rTMS on Ca²⁺ at the synapse. HI-rTMS can initiate action potentials which through glutamate release cause post synaptic calcium influx through NMDA and AMPA receptors, its effect has also been shown to be reliant on VGCCs. In contrast, LI-rTMS causes a modest release of calcium from intracellular stores (although research has not investigated whether ryanodine receptors (RyR) or inositol triphosphate receptors (IP3R) are involved). Both kinds of stimulation will cause an increase in post synaptic calcium however the location, size and frequency may all determine whether the calcium contributes to the induction or the regulation of LTP and LTD. (Adapted from (Rose & Konnerth 2001))

1.3.5 Summary

rTMS can affect a multitude of different systems in the brain depending on the neuron population targeted and the protocols applied. This makes rTMS a flexible therapy option for many disorders as it can not only modulate existing plasticity mechanisms within the brain, but also stimulate the release of various modulatory molecules, which may be beneficial in treating the condition.

1.4 Therapeutic Uses of rTMS

Since its discovery in 1985 rTMS has been trialled as a treatment for a variety of neurological disorders. The first and only disorder for which it is approved by the Food & Drug Administration (FDA) for clinical use is for treatment resistant depression (TRD) (George et al. 2013). Other disorders have trialled rTMS with various successes. Particularly, for the treatment of tinnitus, neuropathic pain and obsessive compulsive disorder, rTMS treatment is prominently researched but not officially prescribed (Wassermann & Zimmermann 2012). Recently, there is increased interest in the effects of rTMS for stroke, schizophrenia, Parkinson's disease and Alzheimer's disease (AD) (Machado et al. 2013). In this thesis I will focus on the treatment of two conditions one where rTMS is well established as a treatment, depression, and one where its use is a relatively new concept, Alzheimer's disease. Both disorders and the implication of rTMS for their treatment will be reviewed below.

2. Depression

Depression is one of the leading causes of disability worldwide. It affects 25% of people at least once during their lifetime (Hendrie & Pickles 2012) and with a mortality rate of 10% it is one of the largest contributors to the worldwide burden of disease (Kern et al. 2012). Major Depression is characterized by the persistence of a depressed mood and loss of interest, this is often accompanied by fatigue, abnormal appetite, irritability and concentration difficulties (Croy et al. 2014; Kern et al. 2012). The severity of these symptoms can affect everyday life for the people diagnosed and even lead to suicide. Different subtypes of depression can be further classified (Benazzi 2006). Atypical, melancholic and anxious depression present different symptoms and can require personalised treatment (Uher et al. 2011). The condition can be easily diagnosed from

these symptoms, but there still lacks a fundamental understanding of the neurobiological causes and the most effective treatments (Krishnan & Nestler 2008).

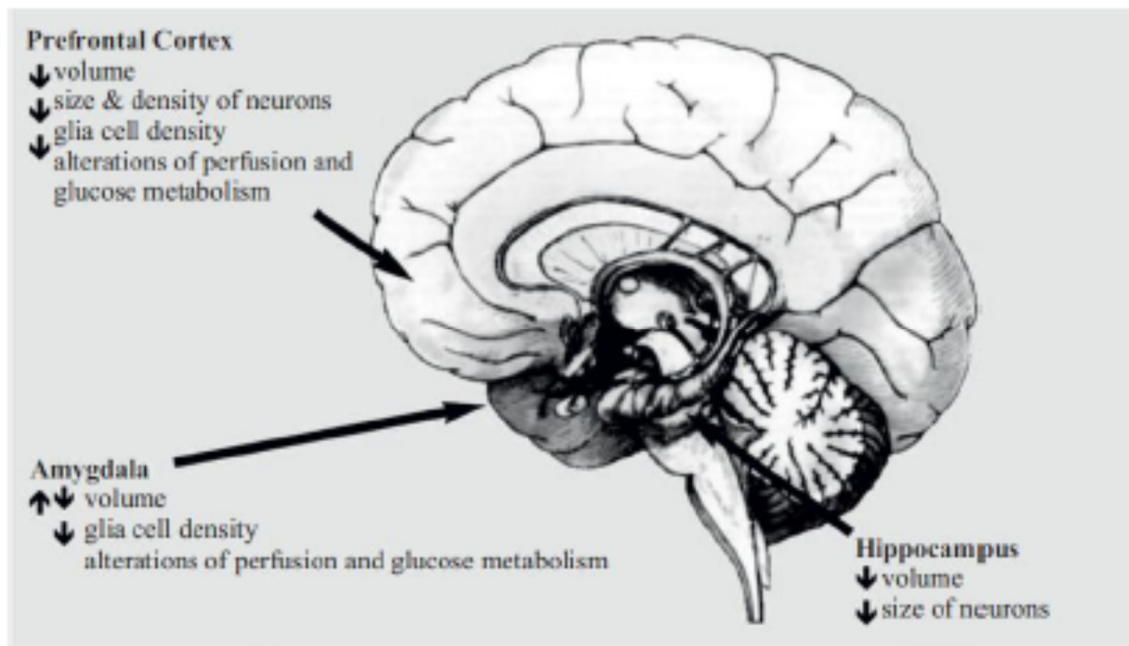


Figure 1.5 Specific areas of the brain involved in depression. Changes have been shown in the prefrontal cortex, amygdala and hippocampus. (Kern et al. 2012)

2.1 Pathology

The exact cause of depression is still unknown; biological factors as well as environmental influences have been implicated, adding to the complexity of this condition (Krishnan & Nestler 2008). Some core brain areas are involved in depression including the prefrontal cortex and the limbic system (Figure 1.5 (Berton & Nestler 2006; George, Taylor & Short 2013; Rajkowska 2003) for meta-analysis see Kempton et al. (2011)). These are the areas of the brain which control a variety of different aspects of daily function, such as processing emotions, memories and stress (Price & Drevets 2010). Additionally, dysfunction of three main biological processes are proposed to cause depression; including changes of monoamine, neuroendocrine and neurogenesis systems (Kern et al. 2012). Unravelling the different contributions of these components to depression is difficult, however it is interesting that evidence has shown rTMS can target all these processes.

2.1.1 Monoamine Changes

The monoamine theory of depression is based on the efficacy of antidepressant medication, such as monoamine uptake or degradation inhibitors, which directly increase the levels of monoamines in the brain and relieve symptoms in some patients

(Trivedi et al. 2006). The main monoamines targeted by these medications are serotonin, noradrenaline and dopamine (Feighner 1999). Therefore, a deficiency in the level of these monoamines was hypothesised as a possible cause of depression. However, studies looking at levels of these monoamines have produced conflicting results as to whether deficiencies are actually seen (Delgado 2000) and moreover the antidepressant effect usually takes weeks to appear therefore it is unlikely to be a simple acute elevation in the level of these monoamines that alleviates symptoms in these patients (Andrade & Rao 2010). Recently, there has been a focus on the changes in structural elements of the neurotransmitter system such as receptor super- and sub-sensitivity and it has been hypothesised these drugs may be restoring a balance in excitation and inhibition of particular neurons (Andrade & Rao 2010; Elhwuegi 2004). It is generally accepted that the original hypothesis that monoamine depletion causes depression is overly simplistic, and the role of monoamines is possibly more complex.

2.1.2 Neuroendocrine Imbalance

Changes to the hormonal system have been investigated as a cause of depression, because stress is often associated with the onset of the disorder (Krishnan & Nestler 2008). The hypothalamic-pituitary-adrenal (HPA) axis is the neuroendocrine control centre of the stress response and hormones in the brain (Goldstein et al. 2011). It is highly regulated, with varying inputs and a strong negative feedback component. Hyperactivation of this system has been recorded in those with depression (Bhagwagar et al. 2005; Kern et al. 2012) and even in non-depressed individuals, elevated cortisol is associated with stressful life events (Van Eck et al. 1996; Wolf et al. 2001). Increased stress hormones including cortisol are known to have disastrous downstream effects, including impacting memory (Buss et al. 2004), fat deposition (Fraser et al. 1999) and most importantly emotional behaviour (Schmidt et al. 1999).

2.1.3 Decreased Neurogenesis

Deficits in neurogenesis have been found in patients with depression using non-invasive indirect measures, such as hippocampal size, estimated by MRI scanning, as well as directly measuring brain regions volumes from histologically stained post-mortem samples (Karege et al. 2005). A decrease in hippocampal volume, which is thought to reflect a reduction in neurogenic capabilities, has been found in patients with depression (Kempton et al. 2011). However, labelling post-mortem tissue, which is a more direct

indication of neurogenesis, has shown varied results. Some studies have shown no differences between depressed patients and controls (Sahay & Hen 2007), while others show a decrease in the amount of newly generated cells in patients with depression (Boldrini et al. 2009). Results from post-mortem data are limited due to small sample sizes and the lack of control for antidepressant use, which may itself alter neurogenesis and confound the results (Boldrini et al. 2009; Malberg et al. 2000). There are also various molecules that are involved in neurogenesis, which can be easily measured, that indicate a deficit in proliferation. For example, BDNF which contributes to the development and survival of newly born neurons, is decreased with depression (Karege et al. 2005; Sen et al. 2008). Although the evidence for the involvement of neurogenesis in depression is conflicting, further research is warranted to clarify the role of newly born neurons in the disorder.

2.1.4 Other Neurobiological Causes

There are other aspects of brain function which are postulated to be responsible for depression. Circadian rhythm and neuroimmunological disruptions are frequently described in depression, and examination of genetic factors to determine hereditary components is a popular and expanding research field (Kern et al. 2012). Nonetheless, it is unlikely that any of these mechanisms would be working alone as there are clear interactions between each of these systems. For example, hippocampal neurogenesis is inhibited by both activation of glucocorticoid receptors (Kim et al. 2004) and antagonism of serotonin receptors (Radley & Jacobs 2002). Determining a comprehensive hypothesis of all these theories together is difficult, but the theoretical framework proposed by Baeken and De Raedt (2011) provides an interesting suggestion (Figure 1.6). Hypercortisolemia, an increase in cortisol levels, disrupts the negative feedback system of the HPA axis, as well as having a neurodegenerative effect on the frontal cortex and hippocampus. The cause of the hypercortisolemia could be enhanced by amygdala activation or a prolonged stress response, incorporating both environmental and neurobiological influences.

A further complication is that it is unclear whether the various changes previously mentioned are the actual cause of depression or just symptoms of a cause somewhere else in the brain. Therefore, to be effective, treatments must address all these possibilities.

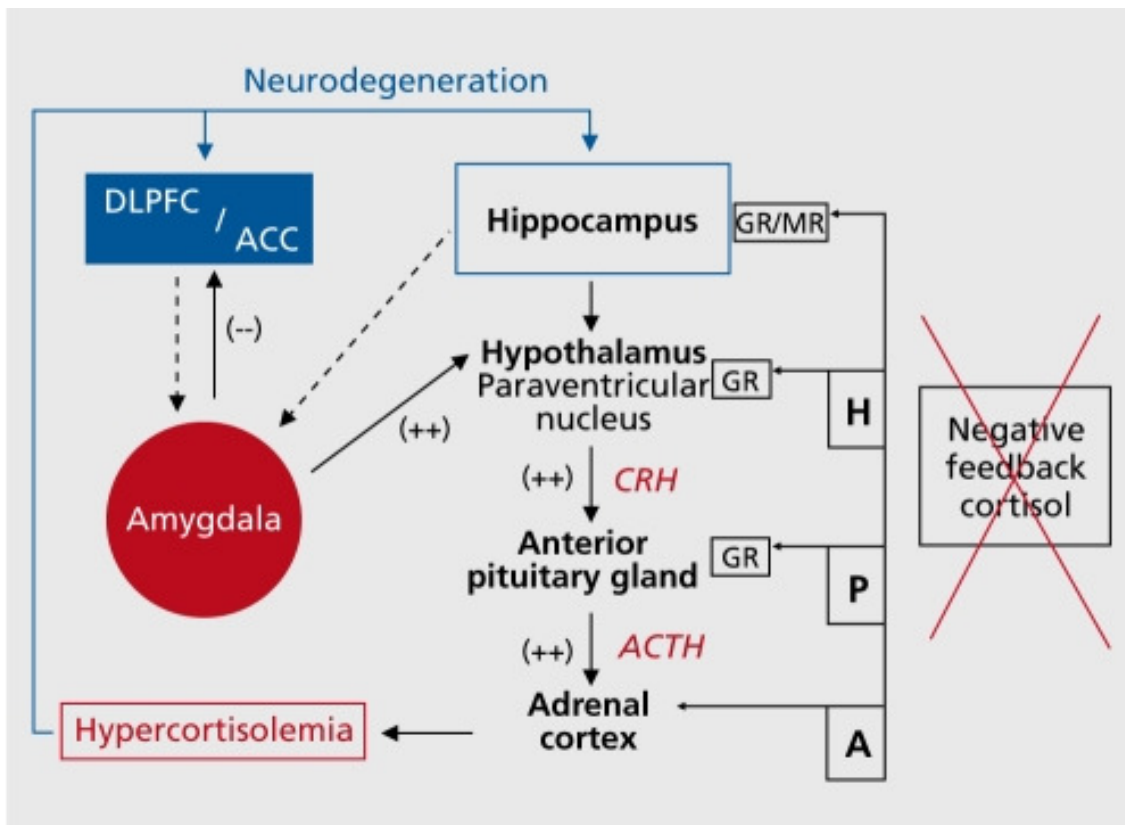


Figure 1.6 Proposed relationship between the limbic system and deregulated HPA axis in depression. Hypercortisolemia caused by either prolonged stress exposure or the disruption of limbic inputs (amygdala hyperexcitability), causes neurodegeneration of various brain structures such as the dorsolateral prefrontal cortex (DLPFC), the anterior cingulate cortex (ACC) or the hippocampus. Loss of the negative feedback control of this system further accentuates the hypercortisolemia (Baeken & De Raedt 2011).

2.2 Current Treatments

There is no lack of treatments for depression. The most common treatment for depression is anti-depressant medication that targets monoamine deficiencies including tricyclic antidepressants and selective serotonin reuptake inhibitors (SSRIs) (Berton & Nestler 2006). Both of these drugs increase synaptic levels of serotonin (as well as noradrenaline for tricyclic antidepressants) by inhibiting membrane transporters (Berton & Nestler 2006). Antidepressant medication has proven to induce remission in a significant portion of patients suffering from depression, and is even more effective when combined with psychotherapy (Cuijpers et al. 2014; de Maat et al. 2007). However, there is debate whether in mild depression this combined treatment provides any beneficial improvement over placebo (Khan et al. 2002a). In addition, publication bias often inflates the significance of these values; an analysis of papers submitted to the FDA showed that a paper analysing antidepressant efficacy was five times more

likely to be published if it showed a significant rather than a negative result (Pigott et al. 2010). Therefore, defining the true efficacy of antidepressant medication is difficult. But regardless of the end statistic, there remains about a third of patients who do not respond to any antidepressant medication (Quitkin et al. 2005).

2.3 Treatment Resistant Depression

People with depression who do not respond to antidepressant medication are termed as having treatment resistant depression (Hendrie & Pickles 2012). The percentage of nonresponse to antidepressants is sometimes estimated as high as 50% (Fava 2003). Specific forms of depression including atypical and melancholic, are also associated with higher treatment resistance (Fava 2003). In these patients, direct neuromodulation is a promising alternative therapy (Holtzheimer & Mayberg 2012). Electroconvulsive therapy (electrical shocks to the brain to induce seizures) has been effective in alleviating this condition but has the major side effect of amnesia (Khalid et al. 2008). As a result, other less severe methods of neuromodulation, including rTMS, have been explored (Holtzheimer & Mayberg 2012).

2.4 rTMS for the Treatment of Depression

In 2008 rTMS was approved by the FDA for use in patients with TRD (George, Taylor & Short 2013). A recent meta-analysis has shown as high as 75% of participants had positive responses, defined as a $\geq 50\%$ reduction in scores on the Hamilton Depression Rating scale, and remission occurring in as high as 50% of patients in another study (Berlim et al. 2014), proving it to be an effective alternative treatment for depression. The theory of the antidepressant ability of rTMS is related to restabilising balance in the limbic system, by targeting specific frontal cortex areas, such as the dorsolateral prefrontal cortex, to affect downstream connected brain structures such as the amygdala (Baeken & De Raedt 2011).

There exists a lack of consistent stimulation parameters for the treatment of depression (Table 1.1). Often, either the left or right dorsolateral prefrontal cortex (DLPFC) is targeted, but that is where similarities between studies end. For treatment of the left DLPFC, high frequency stimulation is used, though this can range from 5-20Hz. A high intensity stimulation is also used but can range from 80-120% of the resting motor threshold. The total number of pulses per session, which has shown to be important for efficacy (Teng et al. 2017), ranges from 800-3000 and the total duration of treatment

from two to seven weeks. A few studies have compared treatment parameters directly to define the most appropriate regime (Bakker et al. 2015; Brunelin 2012; Schulze et al. 2018) but there still lacks a clear consensus. This has resulted in rTMS treatment not being as effective as it could be if the treatment parameters were optimised. Chapter 2 of my thesis will focus on intensity to assess how altering this parameter can affect the outcome of the stimulation.

Table 1.1 Summary of recent studies investigating the efficacy of rTMS for the treatment of depression. All these studies have a range of frequency, intensity, pulses per session and days of treatment, making it difficult to compare the results of each study.

	Frequency (Hz)	Intensity (% rMT)	Pulses per Session	Days of Treatment
Philip et al. (2016)	5	120	3000	36
Concerto et al. (2015)	10	120	3000	20
Benadhira et al. (2017)	10	110	2000	20
Richieri et al. (2017)	10	120	2000	20
Hernández-Ribas et al. (2013)	15	100	3000	20
Zheng et al. (2015)	15	110	3000	20
Hayasaka et al. (2017)	20	95	1000	10
Bakim et al. (2012)	20	80/110	800	30
Baeken et al. (2013)	20	110	1560	20

3. Alzheimer’s Disease

As our population ages there is an increasing range of conditions which are debilitating and detrimental, cost the economy millions of dollars every year but also decrease the standard of living for those in the last years of their lives. Alzheimer’s disease (AD) is one of the most common neurological conditions in the elderly, affecting 35 million individuals worldwide (LaFerla & Green 2012). The condition was first described in 1906 by Alois Alzheimer in which he noticed senile plaques in the brains of those with dementia (Alzheimer 1906). AD can be classified as sporadic AD (SAD), occurring in later years of life or familial AD (FAD), which is the early onset, genetic form. Though AD is such a prevalent condition affecting the elderly, the exact underlying cause of this dementia is yet unknown (LaFerla & Green 2012).

3.1 Memory Decline in Alzheimer's Disease

Those with AD experience a progressive loss in cognitive functions over the course of the disorder. Generally, the first stage of Alzheimer's disease is mild cognitive impairment (MCI). This manifests as small interferences such as linguistic, attentional, and visual deficits, as well as impairments in abstract reasoning (Carlesimo & Oscar-Berman 1992). Mild cognitive impairment is not always indicative of AD and can often be a precursor to other forms of dementia. However, interferences in particular forms of memory can provide indications that AD is developing (Chan et al. 2016). As the disease progresses memory functions that involve medial temporal lobe structures such as declarative and working memory are strongly affected while non-declarative memories such as procedural memory are relatively unchanged until the very late stages of the disease (Gold & Budson 2008; Karantzoulis & Galvin 2011).

Episodic, semantic and spatial memory loss are the most well-defined losses in AD. Episodic memory is the remembering of autobiographical details of one's life and the temporal and spatial context of these moments (Tromp et al. 2015). The deterioration of this memory type is considered one of the first indicators of AD as deficits appear from very early on in the disease (Karantzoulis & Galvin 2011). Semantic memory is the memory of facts and AD patients experience a progressive loss in semantic memory function over the course of the disease (Hodges & Patterson 1995). Those with AD have difficulty in a variety of semantic knowledge tests, including naming objects, places and people (Adlam et al. 2006; Montembeault et al. 2017). Very early in AD, disorientation and getting lost are well documented (Vlček & Laczó 2014), demonstrating a loss in spatial memory. Spatial memory on its own is hard to define as it often involves both components of episodic and semantic memory (Moscovitch et al. 2005). Spatial memory deficits are specific to this particular dementia and can be used to predict the transition into AD from MCI (Bird et al. 2010; Chan et al. 2016; Pengas et al. 2012).

3.2 Pathology

The memory loss previously described is the major symptom and outcome of AD, but within the brain there are deposits and lesions that can also be used to define the progress of the disorder. These include amyloid plaques, neurofibrillary tangles and brain atrophy (Figure 1.7). Although these biomarkers are consistent in most AD cases, the exact role that they play in the pathology of AD is still unclear as there may be several

other factors, including calcium homeostasis, contributing to the loss of memory in the disorder (Murphy & LeVine III 2010; Popugaeva et al. 2017).

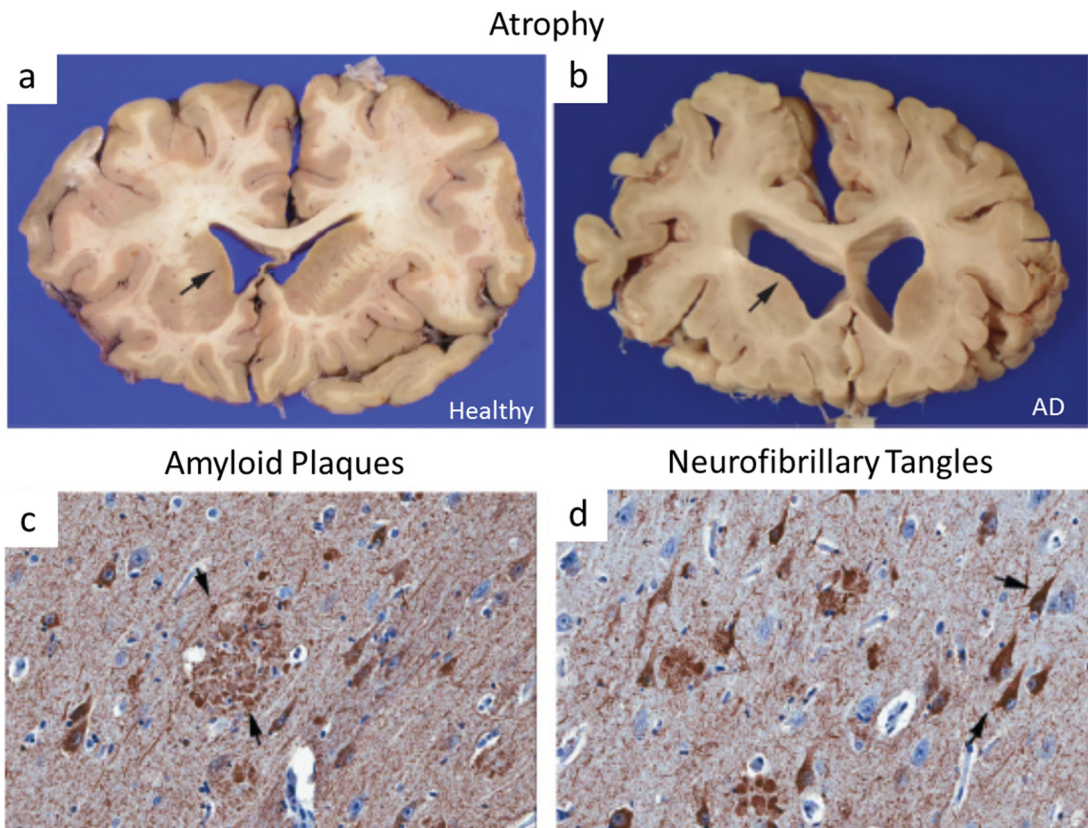


Figure 1.7 Neuropathology of Alzheimer's disease. Alzheimer's disease is easily diagnosed by the presence of three pathological hallmarks. Coronal sections of a brain from a healthy (A) and patient who had AD (B) demonstrates the severe atrophy which occurs in the disorder. The two other hallmarks, amyloid plaques (C) and neurofibrillary tangles (D), can be easily confirmed by post-mortem staining (deposit indicated by arrow). (Vinters 2015)

3.2.1 Atrophy

Atrophy in AD is a well-documented biomarker (Frisoni et al. 2009). As the disease progresses atrophy can be seen in most brain areas, with overall the size of AD brains being 2.5 times smaller than healthy aged individuals (Chan et al. 2001; Ezekiel et al. 2004). As atrophy can be examined with magnetic resonance imaging in humans, it is most researched longitudinal symptoms of the condition. From the earliest stages of AD there is widespread grey matter atrophy across the brain (Frisoni et al. 2009), however, there also exists severe volume reductions in specific subcortical structures which follow a hierarchy of loss, first in the hippocampus and amygdala, and then in the thalamus and putamen (Roh et al. 2011). In the cortex, research has identified progressing atrophy in the precuneus, medial temporal and posterior parietal regions at the earliest stages of the disease, extending later to the prefrontal, sensory and motor cortices (Buckner et al. 2005; Frisoni et al. 2009).

Hippocampal atrophy is the most used biomarker of AD, many research studies have shown its progression across the course of the disease and its relationship to the severity of memory decline (Deweert et al. 1995; Jack et al. 2002; Jack et al. 2000; Schuff et al. 2009). In many studies hippocampal volume is reduced between 15-40% from healthy controls and its reduction is even distinguishable in the early MCI stages of AD (Ridha et al. 2006). Though it is a profound marker of AD, hippocampal atrophy is also prominent in many other conditions and therefore lacks specificity in this disorder (Pini et al. 2016). Amygdala atrophy has also been shown to correlate with disease severity, though it may be more related to anxiety and irritability symptoms in those with AD (Poulin et al. 2011).

3.2.2 Amyloid Plaques

Amyloid plaques are deposits seen in the brain of those with Alzheimer's disease. They consist of 40- to 42-residue β -amyloid ($A\beta$) peptides, which are produced from the cleaving of amyloid precursor protein (APP) by the transmembrane proteins β - and γ -secretase (Murphy & LeVine III 2010). The plaques were first visualised in post mortem tissue from AD patients, but now using imaging techniques plaques can be imaged during the course of the disorder (Rodrigue et al. 2009). The presence of amyloid plaques is seen before behavioural symptoms of the disease begin to manifest and show a distinct pattern of deposition which is reproducible amongst most people with AD (Engler et al. 2006; Grothe et al. 2017).

Plaques as well as diffusible $A\beta$ are toxic to various processes in the brain. Increased amyloid load causes the deterioration of synapses, neuronal loss and increased oxidative stress (Oakley et al. 2006; Reddy & Beal 2008; Xie et al. 2013). Despite the well described harmful effects of amyloid, the exact relationship of amyloid to AD memory loss is elusive, as the level of this protein in the brain only weakly correlates to the level of cognitive decline (Giannakopoulos et al. 2003; Murphy & LeVine III 2010). Furthermore, studies which have used immunisation against the toxic 1-42 β -amyloid peptide showed its removal made little improvement to cognitive decline (Holmes et al. 2008).

3.2.3 Neurofibrillary Tangles

Dense aggregates of the microtubule-associated protein, tau, known as neurofibrillary tangles (NFTs) are also seen in the brain of AD patients. Within neurons, tau promotes the assembly of tubulin into microtubules, but when this protein becomes

hyperphosphorylated it dissociates from the cytoskeleton and aggregates (Kopeikina et al. 2012). Levels of NFTs correlate better with the severity of the disorder and neuronal loss than A β plaques, and the presence of NFTs follows a predictable pattern in most AD patients (Giannakopoulos et al. 2003). Their presence is first seen in entorhinal structures, then the limbic system (hippocampus, amygdala and thalamus) and finally neocortical areas (called isocortex in the Braak classification (Braak & Braak 1991)).

Not only are the levels of NFTs well correlated to disease progression, but the neurodegenerative effects of both these NFTs and soluble tau are evident. When tau dissociates it causes destabilisation of microtubule processes which are involved in neuronal transport, particularly of mitochondria (Kopeikina, Hyman & Spires-Jones 2012). This can result in decreased cell function and synapse loss (Morfini et al. 2009). Neurofibrillary tangles can cause disruption of the neuronal membrane integrity which can have significant effects on the homeostasis of the neuron (de Calignon et al. 2009; Kopeikina, Hyman & Spires-Jones 2012).

3.2.4 Calcium Dysregulation

Changes to calcium levels and to calcium homeostasis within neurons have been discovered in patients with AD and in models of the disease. Within a neuron, calcium concentrations are carefully balanced across the cellular membrane and within the ER (Kumar et al. 2009) and disruptions to this balance can lead to neurodegenerative effects (Popugaeva, Pchitskaya & Bezprozvanny 2017).

It is inherently hard to measure calcium levels within neuronal cells in living humans. The changes involving calcium are transient, therefore measuring these differences in post mortem tissue is also not possible. Cultures of cells taken from AD patients have enabled real time measurements of calcium changes. Although cultured fibroblasts are not neurons, abnormalities in their calcium metabolism may reflect similar abnormalities in brain cells. Resting levels of calcium remain unchanged but when induced, the increase in calcium was higher in cells cultured from AD patients than healthy controls (Ito et al. 1994; Jaworska et al. 2013; Řípová et al. 2004). These results show a dysregulation in the calcium homeostasis within these AD cells.

The mechanisms of this calcium dysregulation are currently investigated. Proteins with mutations in FAD are known to be related to calcium processes and have a regulatory role in maintaining calcium homeostasis at the ER, either through interactions with

receptors or possibly acting as a calcium leak channel on the ER membrane (Stutzmann et al. 2006; Tu et al. 2006). Both A β and tau pathologies can also be linked to calcium abnormalities. Increased A β disrupts calcium homeostasis by causing internalisation of NMDA or AMPA receptors (Hsieh et al. 2006; Snyder et al. 2005a) and also downregulates calcium sensor proteins such as stromal interaction molecule 2 (STIM2) (Popugaeva et al. 2015). Calcium itself can promote the phosphorylation of tau leading to the formation of neurofibrillary tangles and tau itself can also cause increased calcium (Gómez-Ramos et al. 2006; Yamamoto et al. 2002). Therefore, calcium changes provide a possible mechanistic link between the different pathological marks of AD.

3.2.6 Convergence of Theories: Synaptic Loss

One common feature that all these changes can lead to is synaptic loss, which is a major contributor to the cognitive decline in AD. In animal models of AD, severe loss of LTP is experienced in the hippocampus (Auffret et al. 2010), but in humans it is not possible to directly measure LTP or LTD in the hippocampus. However in cortical areas, decreased LTP has been demonstrated for those with AD (Di Lorenzo et al. 2016; Koch et al. 2012; Koch et al. 2016). In addition, changes to structural components of synapses can be measured in the hippocampus in post mortem studies. Synaptic abnormalities were first described in 1967 (Gonatas et al. 1967) and have now been well documented with reductions in synaptic number correlating well with cognitive decline (Scheff & Price

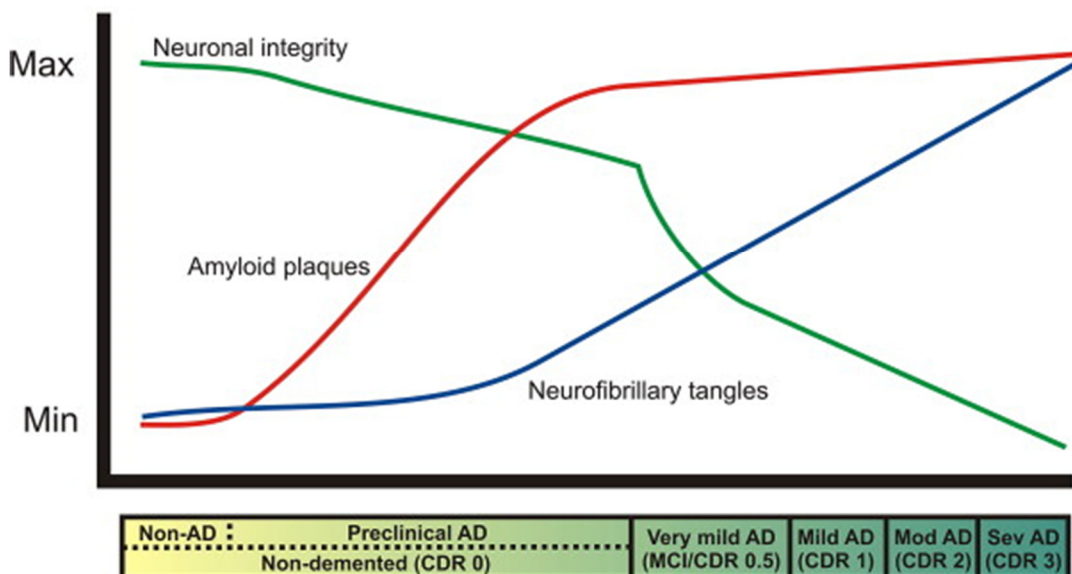


Figure 1.8 Graphical representation of the appearance and progression of AD symptoms. Including neuronal integrity (synaptic changes), amyloid plaques and neurofibrillary tangles. Amyloid plaques are the first symptom to show, even before the diagnosis of mild dementia and reach almost peak deposit levels before high rates of cognitive decline. Both neurofibrillary tangles and neuronal integrity have a better correlation with the development of the disorder (Tarawneh & Holtzman 2010).

1993; Scheff et al. 2006; Terry et al. 1991). Synaptic abnormalities first occur at the MCI stage (Scheff et al. 2006). This demonstrates that synaptic loss follows the progression of the disorder better than other neurological markers (Figure 1.8), which is expected due to the central role that synapses have in learning and memory. Though the main contributor to this synaptic dysfunction is unknown, targeting this synaptic failure may provide a means to delay the onset and improve functions for those with AD.

3.3 Current Treatments

Currently there is no definitive treatment for people with AD. Cholinesterase inhibitors can be used to delay the degradation of acetylcholine and to enhance cholinergic transmission, and NMDA inhibitors are also used to reduce neuronal excitotoxicity (Yiannopoulou & Papageorgiou 2013). Both these treatments are approved for use in patients with AD and although small improvements in cognitive outcomes are seen, benefits are variable and there is a variety of side effects concurrent with these medications (Yiannopoulou & Papageorgiou 2013). Therapeutic interventions currently being investigated are targeting A β and tau accumulation. Amongst the drugs that have made it to late phase clinical trials, improvements in cognitive decline have been disappointing, but there are still many compounds yet to be trialled (Yiannopoulou & Papageorgiou 2013). Alternative therapies to pharmacological interventions have gained interest over recent years. As evidence suggests that AD is defined by the loss of synaptic connections and neuroplasticity, potential treatments that could induce synaptic and neuronal plasticity may be appropriate to target the cognitive decline, one treatment therefore could potentially be rTMS.

3.4 rTMS for the Treatment of Alzheimer's Disease

For patients with AD, the use of rTMS is still in clinical development, unlike depression where its use is approved to treat the disorder. The main mechanism of rTMS is modulation of plasticity and strengthening brain connections (Wang et al. 2014b). So potentially this treatment could affect the disrupted plasticity in AD. Overall, studies have shown that rTMS can significantly improve cognition in patients with AD and dementia (for meta-analysis see Wing et al. (2018)). Research has shown it is more effective in early AD than in a more developed disorder (Ahmed et al. 2012; Padala et al. 2018). However, most studies target the DLPFC which is also the target used for treating depression (Ahmed et al. 2012; Cotelli et al. 2011; Cotelli et al. 2006; Devi et al.

2014; Padala et al. 2018). The DLPFC is associated with many brain functions, including both memory and mood regulation (Kumar et al. 2017). There has been speculation that the improvements seen in cognition may be partly due to improvements in mood in these patients (Rutherford et al. 2013). In AD studies which target the DLPFC negative neuropsychological symptoms such as apathy and depression were alleviated in conjunction with improvements in cognition after rTMS (Ahmed et al. 2012; Nguyen et al. 2017; Padala et al. 2018). A few studies have explored targeting alternative structures including the precuneus, temporal cortex and the inferior frontal gyrus with interesting results (Anderkova et al. 2015; Koch et al. 2018; Zhao et al. 2017b). They showed targeting these specific cortical areas could alleviate specific deficits associated with AD, such as improving episodic memory after stimulating the precuneus or attention after targeting the inferior frontal gyrus (Anderkova et al. 2015; Koch et al. 2018). This demonstrates the potential rTMS can have as a treatment for AD, but more research is needed to elucidate the most effective stimulation site to target the pathology of the disorder.

4. Animal Models

Given the concerns and variability of rTMS treatments for depression and AD, animal models are potentially useful and important to assess the underlying neurological changes being induced, with the aim of increasing clinical efficacy. However, there are limitations for translating rTMS between humans and animal models, as well as accurately representing specific aspects of these neurological conditions within the animal models. This thesis will examine some of these translational issues to acknowledge and potentially address some of these problems.

4.1 Animal Models of rTMS

Investigating rTMS in animal models has been essential to decipher the mechanisms of action of this treatment and preclinical studies may also prove beneficial to compare treatment efficacy between different stimulation parameters. However, methodological differences between human and rodent rTMS could affect accurate comparisons between the two.

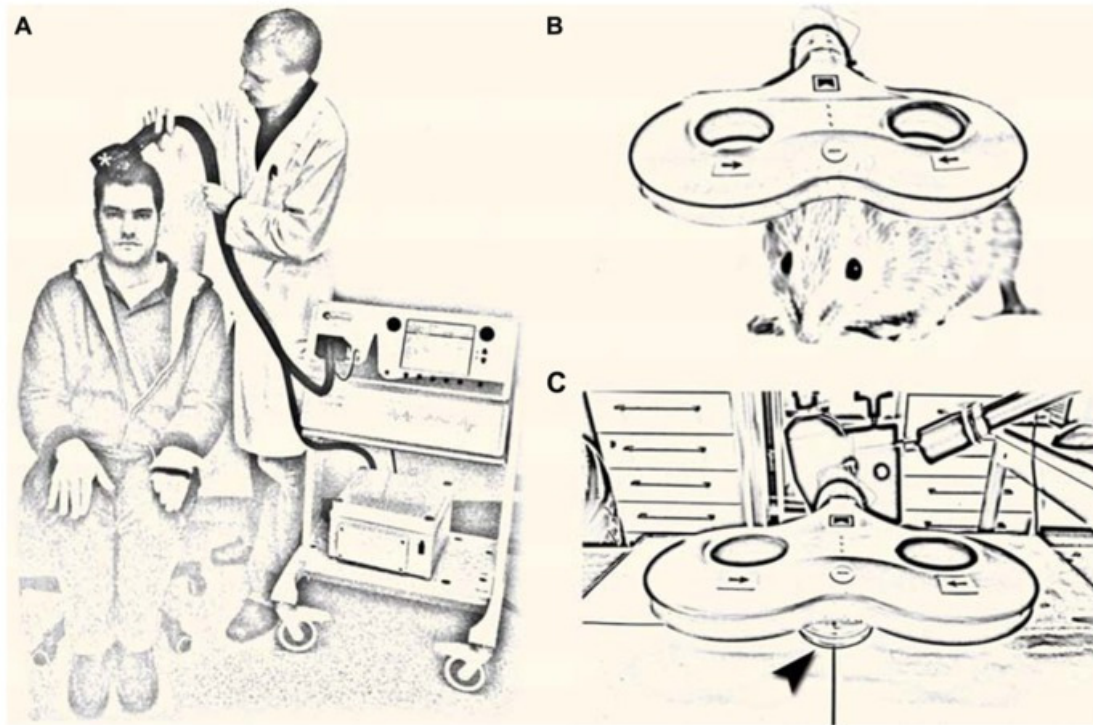


Figure 1.9 Example of human rTMS coils used in animal and *in vitro* studies. As demonstrated in the figure, the same head to coil ratio is not produced when using similar rTMS coils to treat humans (A) or rodents (B). In an *in vitro* setting (C) rTMS coils are used to stimulate the neurons directly. (Müller-Dahlhaus & Vlachos 2013).

4.1.1 Coil Size

A major translational issue between human and rodent rTMS is the coil to head size ratio. The use of human coils is impractical on rodents and *in vitro* studies, as the focality of human rTMS procedures cannot be replicated (Figure 1.9). Many studies have expressed the need for smaller rTMS coils because of the difference in the brain size of rodents compared to humans (Funke & Benali 2010; Post & Keck 2001; Wyckhuys et al. 2013).

Recently, two separate coils have been developed that measure 8mm in diameter meaning they can deliver stimulation to the rodent brain that is more focal than stimulation achieved by human coils (Rodger et al. 2012; Tang et al. 2016b). However, due to engineering constraints these coils can deliver only low intensities of stimulation. One coil design which produces a stimulation intensity of approximately 90mT termed medium intensity rTMS (MI-rTMS) causes plasticity in the motor cortex (Tang et al. 2016a). Despite the very low intensity (Human rTMS is typically delivered at 1-2 T), the smaller distance between coil and brain in rodents compared to humans means that the intensity at the cortical surface approaches that used in human clinical treatment. The other type of coil, which delivers a low intensity stimulation (LI-rTMS) of around 12mT

has also been shown to induce cortical plasticity, but only in the visual system (Makowiecki et al. 2014; Poh et al. 2018; Rodger et al. 2012). The very small size and light weight of these coils has allowed them to be used in animals concurrent with a behavioural task (Poh et al. 2018). Using these small coils in animal models means the stimulation better reproduces the focal magnetic stimulation used in humans, and furthermore the range of available coils enables a comparison of different intensities. These tools will be explored in chapter 2 to determine whether different intensities of rTMS stimulation cause different outcomes.

4.1.2 Anaesthesia

Most animal studies of rTMS do not consider the importance of having the animal awake or anaesthetized. In some studies a semi-targeted stimulation can be induced with larger sized coils if animals are placed in a stereotaxic frame to control the location of the focal area and therefore the animal must be anaesthetized (Vahabzadeh-Hagh et al. 2012). However, as rTMS modulates cortical activity, anything that alters this activity, such as anaesthesia, may hinder its effects. This was demonstrated in a study by Gersner et al. (2011) which showed substantial differences in hippocampal BDNF and the AMPA subunit, GluA1, indicators of neuroplasticity, between awake and anaesthetised animals after rTMS. As humans are awake during rTMS treatment, studies in animals which have used anaesthesia may prove to be misleading, with potentially inaccurate results when relating back to the mechanisms of rTMS in a clinical setting. To reduce the confounding effects of anaesthesia this thesis uses awake animals for stimulation.

4.1.3 rTMS in Animal Models of Neurological Disorder

Many animal studies look at rTMS changes in healthy animals and relate these to how rTMS functions in treating disorders. But even in humans, rTMS can influence brain function in individuals with neurological disorders, without affecting healthy controls (Moulier et al. 2016; Şalçini et al. 2018). It is therefore important to examine rTMS effectiveness in preclinical disorder models to see how the treatment specifically affects the brain with a particular abnormality. There has been some research into the effect of rTMS in animal models of depression and AD, two specific models of depression and AD that have been used in this thesis will be reviewed below.

4.2 Animal Models of Depression

Modelling depression in animals is inherently difficult. The symptoms that are used to diagnose the disorder in humans are hard to detect in animals without specific behavioural testing. As previously discussed, the exact cause of depression is still unknown, and both environmental and neurological factors can influence the onset and persistence of the disorder (Nestler et al. 2002). Animal models have attempted to replicate these influences. These can fall under two broad categories, environmental manipulation models and predisposition models. Environmental manipulation models attempt to replicate the external events that may lead to the development of depression; such as chronic stress or sleep deprivation (Abelaira et al. 2013). Predisposition models examine depression that is not preceded by extensive environmental stressors, instead the model uses a biological factor to cause onset of the disease. One method is through selective breeding for specific symptoms, such as the Flinder's sensitive rat line (Willner & Mitchell 2002), but it is the surgically induced olfactory bulbectomy (OB) model of depression that is the focus of this thesis.

4.2.1 Olfactory Bulbectomy

The olfactory bulbectomy model has been thoroughly developed since its first use in 1975 (Cairncross et al. 1975). In rodents it involves the bilateral surgical removal of the olfactory bulbs (Roche 2011). This surgery allows for a controlled manipulation, with the ability to be consistently replicated between animals. Olfactory bulbs have strong downstream connections to the limbic system that control emotions (Sokolowski & Corbin 2012). Recent studies have shown the relevance of the olfactory bulbs in humans with depression. Patients with acute depression have significantly smaller olfactory bulb volumes (Negoias et al. 2010) and reduced activation from electrophysiological recordings from olfactory processing areas (Croy et al. 2014). These results provide a compelling link between this animal model and depression in humans.

4.2.1.1 Behavioural Changes post Olfactory Bulbectomy

Behavioural tests are vital in animal research into drugs and therapies for treating depression. Though symptoms of depression cannot be measured in animal models by expression of emotions and mood scales as they are in humans, tests can be used to assess specific behaviours in rodents that can be related to human depression.

Open Field Test

The open field test can be used to define different behavioural outcomes in mice. It consists of an open area in which the rodent is placed and the distance covered over a certain period of time is recorded (Roche 2011). This test has been previously described in mice after OB. The increased activity in this test is considered a characteristic behavioural outcome of the surgery (Roche 2011) and has been shown in many studies (Hellweg et al. 2007; Jarosik et al. 2007; Licht et al. 2010; Otmakhova et al. 1992; Zueger et al. 2005). However this hyperactivity is only observed when the open field is presented as a stressful situation, with an illuminated area, and contrasted walls and floors (Mucignat-Caretta et al. 2004; Roche 2011). Chronic antidepressant treatment has been used to reverse this hyperactivity, for example fluoxetine, a common SSRI, has been shown to be effective in Swiss mice (Machado et al. 2012).

Forced Swim Test

The forced swim test is one of the most commonly used behavioural tests to assess depression in animals (Borsini & Meli 1988). The rodent is placed into a cylinder of water and their activity over a certain period of time is assessed, commonly recorded as time spent immobile (Bogdanova et al. 2013). Rodents being assessed after chronic mild stress will become immobile in a shorter period of time, seen as an indication of behavioural despair (Sun et al. 2011). However, after OB surgery in mice hyperactivity in this test is reported (Linge et al. 2013; Mucignat-Caretta, Bondi & Caretta 2004; Mucignat-Caretta et al. 2006), this reflects behaviours in the open field test and may represent an agitated form of depression (Roche 2011). This symptom of depression is often accompanied with anhedonia and anxiety symptoms which are also present in the OB model of depression (Freitas et al. 2013; Machado et al. 2012; Zueger et al. 2005).

Variables which can affect the behaviour in the forced swim test include water temperature, luminance, odour of other animals and combinations with other behavioural tests (Bogdanova et al. 2013). Repeated exposures to the forced swim test can also induce a behavioural despair phenotype in mice, but this is only seen with reoccurring daily exposure (Sun et al. 2011). Extended periods of time between each test does not produce this effect and therefore paired data can be obtained (Vazquez-Palacios et al. 2004).

Other Behavioural Tests

Memory and cognitive impairments can be assessed using tests such as the T-maze or the Morris Water Maze. Deficits in these abilities are seen after OB surgery presenting as a lack of preference in the T-maze (Zueger et al. 2005) and a difficulty in finding the platform in the water maze (Mucignat-Caretta, Bondí & Caretta 2006). Aggressive behaviours, avoidance tests and maternal behaviours have all also been shown to be affected after OB surgery in mice (Jarosik et al. 2007; Mucignat-Caretta, Bondi & Caretta 2004; Mucignat-Caretta, Bondí & Caretta 2006; Roche 2011). The behavioural changes following OB are still not fully understood in mice, but they are reproducible and able to be modulated by antidepressants, providing a good experimental model of depression.

4.2.1.2 Neurobiological Changes after Olfactory Bulbectomy

It is important to document not only the behavioural changes observed following OB to test the efficacy of treatments for depression, but also the neurobiological changes. The fact that anosmia alone cannot induce the behaviours observed indicates that neurobiological changes that extend beyond the loss of smell must underpin these depression-like symptoms (Mar et al. 2000). Because the OB model reflects specific behavioural symptoms of depression, any neurobiological changes identified in the model may be useful in deciphering the development of specific depression subtypes.

Structural Modifications

Post OB surgery, brain regions with direct connections to the olfactory bulbs are structurally rearranged and undergo atrophy. The piriform complex has reduced cell density following OB surgery; this loss was prevented with tricyclic antidepressants (Jarosik et al. 2007), as well as decreased dendritic branching (Morales-Medina et al. 2013a). Reorganization of dendrites and decreased branching was also observed in the entorhinal cortex, but not in the prefrontal cortex, intra-limbic cortex or nucleus accumbens (Morales-Medina et al. 2013b). These structures are the main targets of the olfactory bulb, and their degeneration may be a direct result of reduced input from this structure (Song & Leonard 2005). Neuronal degeneration in these structures inevitably leads to decreased output and may underpin structural changes observed in downstream areas such as the hippocampus and amygdala (Morales-Medina et al. 2013a), habenular nuclei, which mediate neurotransmitter systems (Oral et al. 2013), and the dorsal raphe nuclei in mice (Nesterova et al. 1997).

Neurotransmitter

Neurotransmitter changes also occur after OB surgery in rodents. The synthesis of serotonin in the raphe nuclei and extracellular serotonin in the basolateral amygdala and dorsal hippocampus is decreased after surgery (van der Stelt et al. 2005; Watanabe et al. 2003). Noradrenaline has also been shown to be reduced following bulbectomy, specifically in the ventral and dorsal striatum (Masini et al. 2004; Van Riezen & Leonard 1990). Conversely, it has been reported that there is an increase in dopamine turnover and function in the ventral and dorsal striatum (Hellweg et al. 2007; Masini et al. 2004).

In addition, glutamate, which is linked to hyperactivity, was found to be increased in the striatum in response to stress after OB (Ho et al. 2000). However, decreased NMDA receptor expression has been found in the amygdala (Robichaud et al. 2001) and other glutamate receptor subtypes were also decreased in the hippocampus (Wieronska et al. 2008). After OB surgery there is also an increase in GABA receptors in the frontal cortex (Dennis et al. 1994). This suggests region specific changes to both glutamatergic and GABAergic systems after OB, especially in response to stress. These neurotransmitter changes are a possible mechanism behind modified activity levels in OB mice (Wieronska et al. 2008).

Neuroendocrine

As abnormal reactions to stress are very prominent in the behaviours observed following OB it would be expected that there would be changes to the neuroendocrine system. The surgery has no effect on basal levels of corticotropin releasing hormone and ACTH (Holmes et al. 1998; Marcilhac et al. 1999a; Marcilhac et al. 1999b), but increases basal arginine vasopressin and corticosterone (Cairncross, King & Schofield 1975; Machado et al. 2012; Marcilhac et al. 1999a; Marcilhac et al. 1997). Under stressful conditions additional changes are observed to the levels of these circulating hormones. After an acute stress, OB rodents have significantly increased corticosterone and ACTH responses compared to controls (Cairncross, King & Schofield 1975; Marcilhac et al. 1999b), indicative of an overactive HPA system. Interestingly only the increase in ACTH is attenuated by fluoxetine treatment (Marcilhac et al. 1999b). Examining all the changes together, OB results in a hyper-excited HPA axis in response to stressors, supporting the hyperactive behavioural responses.

Neurogenesis after Olfactory Bulbectomy

There is a decrease in proliferation abilities of the hippocampus following OB, which is consistent with data in human depression. In the hippocampus, decreases in both cell proliferation (Jaako-Movits & Zharkovsky 2005) and survival (Jaako-Movits et al. 2006; Keilhoff et al. 2006; Shioda et al. 2010) have been reported following bulbectomy. The decrease in both proliferation and survival was shown to be reversed by chronic antidepressant treatment using the SSRI citalopram (Jaako-Movits et al. 2006) and the tricyclic imipramine (Keilhoff et al. 2006). Interestingly, an increase in cell proliferation (BrdU staining) in the amygdala was also found (Keilhoff et al. 2006).

4.2.2 rTMS and Animal Depression Models

To date, there have been only a few investigations into the effects of rTMS in animal models of depression. Studies have shown its effectiveness in reversing symptoms in chronic mild stress models (Feng et al. 2012; Sachdev et al. 2002; Sun et al. 2011). One study examined the effect of changing the parameters of stimulation showing higher frequencies to be more effective (Sachdev et al. 2002). In the rat olfactory bulbectomy model, rTMS attenuated the behavioural changes (Vieyra-Reyes et al. 2008). However, the rTMS condition used in this study was a 60 Hz stimulation for four hours a day, which is unlike conditions used in humans and may in fact be harmful (Berlim et al. 2014). Additionally, most of these studies used an antidepressant to compare the effectiveness of the rTMS treatment, whereas in humans, rTMS treatment is only used in cases where antidepressants do not have an effect. Therefore, to be clinically relevant, studies should be assessing the effectiveness and mechanism of action of rTMS in depressive models that show resistance to antidepressants.

4.3 Animal Models of Alzheimer's Disease

Typically, reproducing AD in animal models has proven difficult as the exact cause of AD is still unknown. Animal models rely on translating the known pathology of AD into animals and assessing the effects on memory processes. The most common animal models of AD rely on transgenic manipulation, to either replicate mutations found in those with FAD or to cause an overproduction of A β and tau related proteins to produce a plaque and tangle pathology (LaFerla & Green 2012).

4.3.1 FAD mutations

The study of the early onset FAD has led to the classification of three genes in which mutations relate to the disorder: APP, Presenilin 1 (PS1) and Presenilin 2 (PS2). Of particular interest is PS1, which contributes to 78% of the FAD mutations (Zou et al. 2014). PS1 is a major component of the APP cleaving protein γ -secretase, but its involvement in AD pathology is postulated to exceed this one function. Presenilin's are also involved in γ -secretase independent functions including Wnt/catenin regulation, lysosomal proteolysis, calcium homeostasis and synapse regulation (Ho & Shen 2011).

Mutations found in patients with FAD have been replicated in mouse models using knock in mutations. These mutations benefit from the normal production of a mutated protein as opposed to overproduction. While PS1 knock in mutations do not induce amyloid plaque pathology, the overall tissue load of soluble A β is increased (Sun et al. 2005). These models can help us determine an involvement of PS1 in AD, independent of its involvements in production of A β aggregates. These models have been essential in developing ideas of AD not related to A β plaques concerning the loss of synaptic connections and cognitive decline experienced in the disorder. One model is the PS1M146V knock in (KI) model with the human M146V mutation inserted into the PS1 (presenilin-1) genome. Mice with this mutation produce abnormal PS1 (Guo et al. 1999).

4.3.2 PS1M146V KI mice

The PS1M146V mutation was first used in a knock in model in 1999 (Guo et al. 1999) and since then various neurobiological and behavioural changes have been observed which have been vital in understanding the pathology of AD. Compared to other models that have more than one genetic abnormality to induce a plaque and tangle pathology, this model enables analysis of the inherent causes of the condition.

4.3.2.1 Behavioural Changes

Assessing behavioural changes in rodent models of AD is important as they can be used to look at the effectiveness of treatments on specific aspects of cognitive decline and relate changes in behaviour to potential causes of the disorder. Also developing an understanding of other neuropsychological changes is important as these are often correlated with the cognitive decline in AD.

Spatial Learning and Memory

Spatial memory tests are common in animal models of AD, not only because of their ease of measurement in rodents but also because they are highly relevant to the pathology of AD. Spatial memory is one of the first memory deficits in AD and is specific to this form of dementia (Bird et al. 2010; Chan et al. 2016; Pengas et al. 2012). The most common test for assessing spatial learning and memory is the Morris Water Maze (Morris 1984). This test involves having the rodent swim to find a hidden platform using external cues as guides to assess allocentric spatial memory. Depending on the severity of the mutations and the age of the rodent there can be a varying level of disorder in this test (Edwards et al. 2014; Murphy et al. 2006). Animals can either have deficits in the learning component; which is the acquisition of the task over the days of training, or the memory component; where a probe trial assesses the memory of the platform location at the end of the training phase (Morris 1984). Specific aspects of memory can be further probed by assessing search strategies over the course of the test (Garthe et al. 2009; Illouz et al. 2016).

In the PS1M146V KI model, mice do develop mild spatial memory abnormalities from 3 months of age in the Morris Water Maze (Sun et al. 2005). Mice only develop a dysfunction in the memory probe component of the test whereas there is no dysfunction in the learning phase of the test. These deficits continue to worsen up to 11 months of age, this is important as it shows these mice have disease progression similar to humans with a worsening cognitive decline. This contrasts to other AD models which can show very severe cognitive dysfunction from as early as 4 months of age (Liu et al. 2015).

Other Types of Memory altered in PS1M146V KI mice

As Alzheimer's disease affects a range of cognitive processes it can also be important to examine other forms of memory. In the M146V model a deficit in contextual fear learning has also been demonstrated (Wang et al. 2004), in other animal models of AD deficits in fear conditioning are equated to amygdala function (Avrahami et al. 2012; Corcoran et al. 2002). Another form of memory of importance is episodic memory; deficits in this particular form of memory are a major consequence of AD (Karantzoulis & Galvin 2011). Object recognition tests are used to assess episodic memory in rodents. These tests can be divided further into the separate components of episodic memory

such as the “What, When and Where” test (Dere et al. 2005). Mice with AD mutations exhibit deficits in these object recognition tests (Bengoetxea et al. 2015), however these tests have yet to be assessed in the PS1M146V model of AD. Working memory is another cognitive component that can be measured, though like the episodic memory test it has never been examined in the M146V model, however, other AD models present deficits in working memory (Stevens & Brown 2015; Wirths et al. 2008).

Neuropsychological Phenotypes

Anxiety and depression are highly correlated with AD (Teri et al. 1999; Usman et al. 2010); and are linked to the severity of the disorder (Lyketsos et al. 2003). Examining anxiety phenotypes in animal models can help establish a profile of the neuropsychological changes associated with AD. Currently no studies have examined anxiety changes in the PS1M146V KI model. However, in other animal models of AD, anxiety has been examined. One test which examines anxiety like phenotypes is the elevated plus maze (Pellow et al. 1985). This test uses defined open and closed zones to assess anxiety. The results are varied for examining anxiety in other AD models. Some report an increase in anxiety (Beauquis et al. 2014), others report no change (Lalonde et al. 2002; Lok et al. 2013; Wright et al. 2013), and some even report a decrease in anxiety compared to wild type (Jensen et al. 2005). One of the major contributing factors to this discrepancy could be the gender of the animals, with research showing that female AD mice are more likely to display an anxious phenotype than males which reflects the higher prevalence of anxiety in female patients (Ruan et al. 2017).

4.3.2.2 Neurobiological Changes in the PS1M146V KI AD mouse

The PS1M146V mouse provides a unique opportunity to examine neurobiological changes that are directly related to a mutation found in those with FAD. These mice do not develop a typical deposit-related pathology, and therefore highlight the contribution other neurological changes can have to the loss of synaptic plasticity in AD.

A β and Tau Pathology

The M146V KI model of AD does not develop amyloid plaque or NFT deposits, but they do exhibit changes to levels of A β fragments. Depending on the cleavage site of APP, different lengths of A β can be formed (Murphy & LeVine III 2010). Studies have shown PS1M146V mice have an increase in A β 1-42 fragments, but no changes in A β 1-40 (Sun et al. 2005). A β 1-42 is the form of amyloid that aggregates to form the deposits in

humans (Bitan et al. 2003; McGowan et al. 2005) and though these mice do not develop plaques, this peptide has been linked to pathological changes independent of plaque formation (Murphy & LeVine III 2010).

Calcium Homeostasis

Mice with the M146V KI mutation have detrimental changes to neuronal calcium homeostasis within neurons. Calcium dysregulation has become an important focus of AD research and PS1 is known to have a regulatory role in maintaining the calcium homeostasis within neurons (Ho & Shen 2011). The mutation disrupts its interaction with ER calcium receptors and its calcium leak channel function (Barrow et al. 2000; Stutzmann et al. 2006; Tu et al. 2006). This leads to elevated ER calcium which causes a cascade of downstream processes that have been postulated to be related to the functional deficits observed in the model (Sun et al. 2014; Zhang et al. 2015b).

Synaptic Plasticity

One of the clear pathological changes in mice expressing the M146V mutation is the effect on synaptic plasticity. Both electrophysiological and structural components of plasticity have been examined in this model. Changes to LTP have been observed which worsen from 6 to 12 months of age in mice, at the later stages this is accompanied by decreased NMDA receptor function (Auffret et al. 2010). Other electrophysiological measures such as after-hyperpolarisation are also affected in the model (Barrow et al. 2000).

Changes to dendritic spines have also been discovered. In mice with the M146V KI mutation there is a shift from stable mushroom spines to thin spines from 6 months of age (Sun et al. 2014). Interestingly, this dysfunction recovered with experimentally increased expression of ER calcium regulator protein STIM2 (Sun et al. 2014). Another study examined the reason for these decreased levels of mushroom spines and found that after an LTP induction normal spine growth did occur in cultured neurons expressing the PS1M146V KI mutation (Zhang et al. 2015a). However, where normal neurons maintained these mushroom spines the mutation resulted in the spines returning to their normal size within hours. This demonstrates that the mutation causes a negative effect on spine maintenance.

Neurogenesis in PS1M146V KI mice

Studies have also examined the loss of neurogenesis in AD models. In the PS1M146V KI model, reduced proliferation was found in the dentate gyrus, though the survival of proliferated neurons was not examined (Wang et al. 2004). Conversely, one study which examined hippocampal morphology and total cell numbers found no effect of the PS1M146V KI mutation on these morphological features (Sun et al. 2005). However, they examined only the CA1 and CA2/CA3 regions, not the dentate gyrus which is more likely to be affected by the loss of neurogenesis.

4.3.3 rTMS in Animal models of AD

There have already been a few studies examining the effects of rTMS in animal models of dementia, with only two looking at the effect in specific AD models. All the studies show a positive effect of rTMS in mouse models of AD, through improved spatial memory functions and increased LTP (Huang et al. 2017; Tan et al. 2013a; Wang et al. 2015). Looking into the mechanisms of action they have shown rTMS reduces soluble A β and plaque load, increases NMDA receptors and increases BDNF (Huang et al. 2017; Tan et al. 2013a; Wang et al. 2015). There are however conflicting results between these studies as to the most appropriate treatment parameters to use. Studies that use a low frequency 1Hz stimulation show improved performance (Huang et al. 2017; Tan et al. 2013b), but in the study by Wang et al. (2015) there was no effect of 1Hz stimulation, rather excitatory frequencies of 10Hz and 15Hz had the best response. Other studies examining mice with a different form of dementia also have conflicting results between the efficacy of excitatory or inhibitory frequencies (Yang et al. 2015; Zhang et al. 2015b). These studies have been vital in looking into some of the mechanisms behind the effect of rTMS in AD, however the conflicting results warrant further exploration as to the most appropriate frequency to use when treating AD with rTMS. In particular, low intensity magnetic stimulation has never been examined in AD mouse models. Both the effect of different frequencies and the use of LI-rTMS in the PS1M146V model is the focus of chapter 3 of this thesis.

5. Thesis Scope and Aims

As demonstrated in this literature review rTMS has the potential to affect many aspects of brain circuitry and function. It can induce plasticity but also alter how the innate plasticity of the brain occurs. These capabilities make it an interesting, novel therapy to

treat different disorders; including both depression and AD. Both these disorders have different pathologies and symptoms but rTMS has the potential to be a treatment option for both, as already established for depression and now being explored in AD. The broad aim of this thesis is to examine the use of rTMS in preclinical models of depression and AD. Specifically, focusing on altering different parameters of the stimulation to see how this influences the outcome and therefore assessing the ability to tailor rTMS to these different conditions. Two studies were conducted to address this overall aim, but they were also able to expand the knowledge of the mechanisms of rTMS and the underlying pathology of specific preclinical models of depression and AD.

In the first study we investigated use of rTMS in an animal model of depression. We aimed to **a)** identify behavioural and biological changes that respond to rTMS compared to an antidepressant as in a clinical setting rTMS is only used to treat patients who do not respond to antidepressants. **b)** Compare different intensities of rTMS to investigate possible mechanisms of rTMS and the relationship to serum metabolites to expand the knowledge of the influence of changing parameters on the antidepressant effect of rTMS.

In the second study we investigated rTMS in an animal model of Alzheimer's disease. We aimed to **a)** investigate whether LI-rTMS could be used as an alternative therapy to improve cognition in AD as this form of stimulation has shown promise in affecting abnormal plasticity in past studies. **b)** Assess whether altering the frequency of the stimulation affects the outcome as frequency has not been explored thoroughly in lower intensity forms of stimulation. **c)** Compare the effect in two different age groups to see how progression affects both the pathology and outcome of the stimulation because in human studies rTMS is more effective in earlier clinical populations.

From these studies we aimed to be able to compare the use of rTMS for both these disorders. This will elucidate mechanisms of action of the stimulation as well as potentially the underlying pathology it is targeting. Overall, this will build an understanding of appropriate treatment parameters and how rTMS can be specifically tailored for each disorder.

Chapter 2:

Medium- and High-Intensity rTMS Reduces Psychomotor Agitation with Distinct Neurobiologic Mechanisms

Currently, preclinical models of both rTMS and depression are limited. Studies in animal models will often use an antidepressant as a benchmark against which to assess efficacy of new therapies. Therefore, the existing framework has limited scope to improve treatments for patients with treatment-resistant depression. Additionally, intensity is known to play an important role in antidepressant outcomes of rTMS but the mechanisms behind the different effects are still unknown.

To address these limitations, in this chapter we used high intensity rTMS as our benchmark, because this treatment is already validated in the treatment of depression in humans. We altered only the parameter of intensity because this parameter is key to understanding the relevance and efficacy of our low intensity animal coils. To attempt to keep other rTMS parameters constant, we selected the frequency (10Hz), number of pulses (1800 per session) and days of treatment (20 days) to match those used in a clinical setting (Table 1.1). The final protocol differed from human treatment protocols only in that it was delivered in a single uninterrupted session over 3 minutes in order to limit stress on the animals. Human treatment requires intertrain intervals to allow for cooling of high intensity coils, but this was not required in our studies due to the lower intensity requirements in the smaller rodent brain.

In this study we first investigated whether the olfactory bulbectomy (OB) model of depression could serve as a model of TRD by comparing the effects of targeted rTMS and an antidepressant. We first examined the behavioural and neurobiologic changes in OB mice with medium-intensity rTMS (MI-rTMS) treatment compared to the antidepressant, fluoxetine hydrochloride. The forced swim test revealed a psychomotor agitation phenotype in this model that responded only to rTMS, and not to fluoxetine, suggesting a model of treatment resistant depression.

We then went on to compare different intensities and the focality of rTMS coils in this model. We assessed how different intensities affected the behavioural outcome and various neurobiological measures related to depression. We also investigated serum

biomarkers, which can be directly compared to human studies. We compared 10Hz rTMS sessions for 3 minutes at intensities of (measured at the cortical surface) 4mT (LI-rTMS), 50mT (MI-rTMS), or 1T (HI-rTMS), 5 days per week over 4 weeks in an OB model of agitated depression. Behavioural effects were again assessed with forced swim test; neurobiologic effects were assessed with brain levels of brain-derived neurotrophic factor (BDNF) and neurogenesis. Peripheral metabolomic changes induced by OB and rTMS were monitored through enzyme-linked immunosorbent assay and ultrahigh pressure liquid chromatography–driven targeted metabolomics evaluated with ingenuity pathway analysis (IPA). The psychomotor agitation induced by OB in the forced swim test was attenuated with MI-rTMS and HI-rTMS, but only MI-rTMS increased brain-derived neurotrophic factor and neurogenesis levels in specific brain regions and HI-rTMS normalized plasma concentration of α -amino-n-butyric acid and 3-methylhistidine. IPA revealed significant changes in glutamine processing and glutamate signalling in the OB model and following MI-rTMS and HI-rTMS treatment. At the end of the chapter unpublished data on the circulating levels of corticosterone and ACTH has been included, highlighting specific effects of HI-rTMS.

Overall, this study was the first to our knowledge to demonstrate an antidepressant effect of rTMS in an animal model where fluoxetine did not have an effect suggesting that the OB model of depression may be a valid preclinical model of treatment resistant depression. This study also contributes to identify potential metabolites for the presence of TRD and the response to rTMS. Importantly, we distinguished between different intensity effects and discuss the potential implications of using either MI-rTMS or HI-rTMS.

Medium- and High-Intensity rTMS Reduces Psychomotor Agitation with Distinct Neurobiologic Mechanisms

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Introduction

Repetitive transcranial magnetic stimulation (rTMS) has been used clinically since 2008 for treatment-resistant major depressive disorder. The virtually infinite parameter space of dosing rTMS (i.e., coil geometry, coil position, focality, intensity, frequency, session length, session number, and brain state) magnifies the challenges of elucidating mechanisms of action and contributes to diverse clinical outcomes. Notwithstanding their limitations, animal and preclinical models are key tools in refining the mechanistic understanding and optimal stimulus dosing approach for rTMS in major depressive disorder. In many rodent models, the focality of human rTMS cannot be appropriately reproduced because even the smallest animal coil stimulates a large portion of the brain (Wyckhuys et al. 2013). However, coils that achieve focality but at a lower intensity enable the mechanistic study of low-intensity rTMS (LI-rTMS). Low-intensity rTMS may modulate cortical excitability in the prefrontal cortex of humans, thereby addressing clinical psychiatric symptoms. Furthermore, preclinical LI-rTMS protocols may model the perifocal effects of standard clinical rTMS (Boggio 2010; Doeltgen & Ridding 2011; Huang & Rothwell 2004; Todd et al. 2006).

The present study examined a mouse olfactory bulbectomy (OB) model to investigate differential effects of rTMS stimulation intensities on depression-related behaviours and neurobiologic characteristics. The OB model was selected because removal of the olfactory bulbs results in modulation of downstream connections with such limbic structures as hippocampus, amygdala, habenular nuclei, and raphe dorsalis, inducing reproducible behavioural changes that are associated with depression (Morales-Medina et al. 2017; Morales-Medina et al. 2013b). In addition, aspects of these behavioural changes, including hyperactivity or psychomotor agitation in the forced swim test (FST) (Linge, Pazos & Diaz 2013; Mucignat-Caretta, Bondi & Caretta 2004; Mucignat-Caretta, Bondi & Caretta 2006), anhedonia (Freitas et al. 2013), neurocognitive impairment (Machado et al. 2012; Zueger et al. 2005), and anxiety (Amigo 2016) reflect the symptoms of agitated depression in humans, which often shows poor responsiveness to pharmacologic treatment (Gili 2012; Lin et al. 2016; McGrath 2008; Parker 1999). We investigated brain markers of plasticity (i.e., brain-derived neurotrophic factor [BDNF] and hippocampal neurogenesis), brain serotonin, and plasma metabolites in an attempt

to better understand how the cellular and molecular effects of rTMS intensity relate to behavioural outcomes.

The study design was motivated by an unmet need for preclinical dose-finding studies of rTMS for treatment-refractory phenotypes and special clinical populations. First, we examined the behavioural and neurobiologic changes in OB mice with medium-intensity rTMS (MI-rTMS) treatment and fluoxetine hydrochloride. We then compared the same outcomes following 3 different intensities of rTMS to gain insight into relevant neurobiologic mechanisms among variable intensities. Finally, we extended our results by analysing serum samples for potential biomarkers of depression-like behaviour in the OB model and recovery induced by rTMS. We hypothesized that high-intensity rTMS would have the greatest behavioural and neurobiologic impact on an OB model of an agitated, treatment-refractory depression.

Table 2.1. Overview of Experiment 1 and 2 Sample Sizes

Experiment 1	FST	5HT		
SHAM/SHAM	n=11	n=5		
OB/SHAM	n=9	n=3		
OB/MI-rTMS	n=11	n=5		
OB/Flu	n=9	n=4		
OB/Veh	n=9	n=4		
Experiment 2	FST	BDNF	Neurogenesis	Metabolomics
SHAM/SHAM	n=11	n=5	n=4	n=4
OB/SHAM	n=12	n=8	n=4	n=9
OB/LI-rTMS	n=16	n=7	n=7	n=11
OB/MI-rTMS	n=13	n=8	n=5	n=10
OB/HI-rTMS	n=15	n=7	n=6	n=7

BDNF, brain-derived neurotrophic factor; 5HT, 5-hydroxytryptamine; Flu, fluoxetine; FST, forced swim test; HI-rTMS, high intensity repetitive transcranial magnetic stimulation; LI-rTMS, low-intensity repetitive transcranial magnetic stimulation; MI-rTMS, medium intensity repetitive transcranial magnetic stimulation; OB, olfactory bulbectomy; Veh, vehicle.

Materials and Methods

Study Overview

To examine behavioural (FST) and prefrontal cortex 5-hydroxytryptamine (5HT) changes we first compared mice treated with rTMS, fluoxetine and their respective controls. These five groups included mice undergoing a sham surgery and receiving sham rTMS

(n=11), mice undergoing OB and sham rTMS (n=9), mice undergoing OB and MI-rTMS (n=11), mice undergoing OB treated with fluoxetine (n=9), and mice undergoing OB treated with a vehicle (n=9). 5HT changes were measured in subsets of these mice. In a second set of experiments, mice undergoing a sham surgery and sham rTMS (n=11), OB mice treated with sham rTMS (n=12), OB mice treated with LI-rTMS (n=16), OB mice treated with MI-rTMS (n=13), and OB mice treated with HI-rTMS (n=15) had FST testing. Frontal cortex and hippocampal BDNF, hippocampal neurogenesis, and plasma metabolomic studies were conducted in subsets of these treated mice. Table 2.1 provides an overview of sample sizes. All post mortem tests and video analyses were blinded.

Study Animals

C57Bl/6j mice (male sex, aged 8 weeks at start of experiment) were group-housed and maintained under a standard 24-hour light-dark cycle (i.e., lights on 0600 and lights off 1800) with ad libitum access to food and water. Each cage contained up to five animals. Mice with aggressive behaviours after OB were moved to separate housing. Housing and bullying were taken into account in statistical analyses. All procedures were approved by the University of Western Australia animal ethics committee (RA03/100/1298) and conformed to US National Institutes of Health guidelines. Experimental design and timeline are shown in Figure 1A.

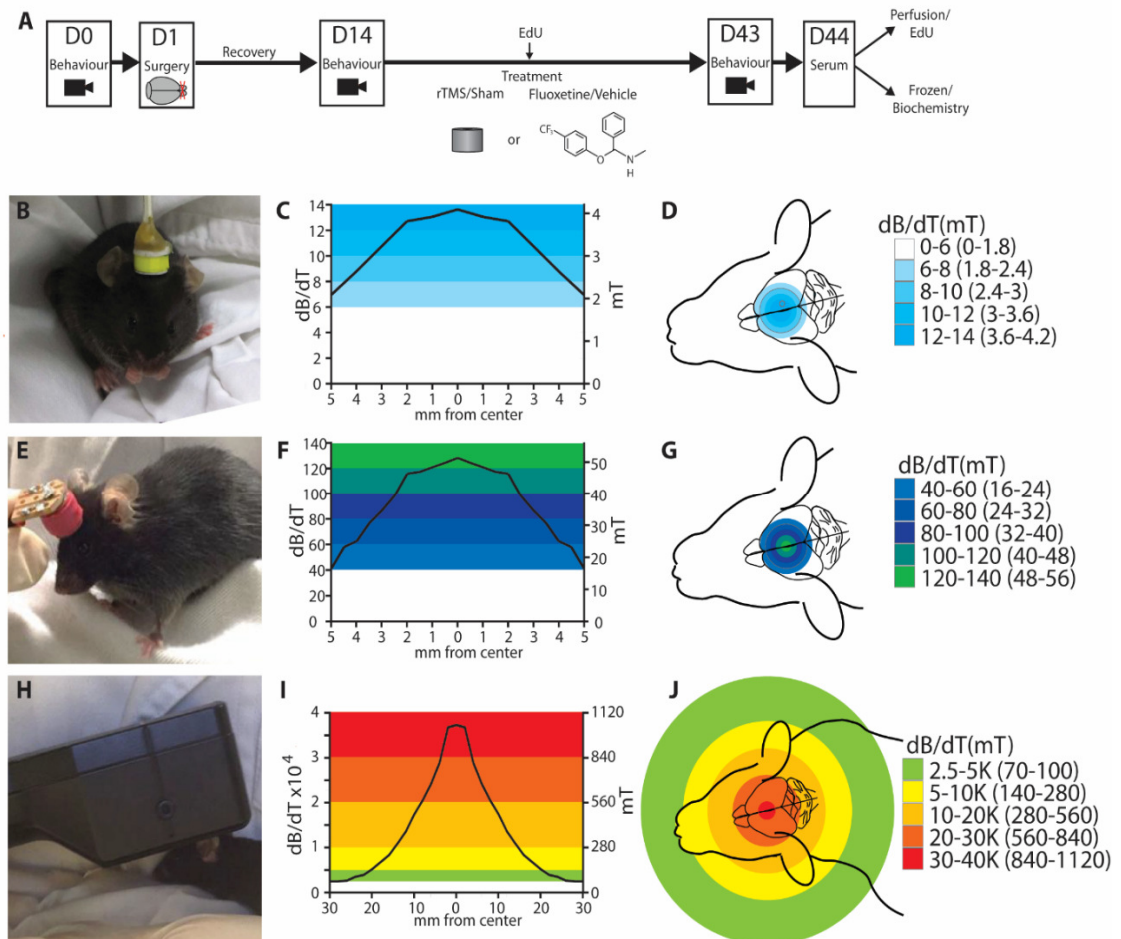
Olfactory Bulbectomy

OB was performed according to several previously established surgical protocols (Amigo 2016; Hirose et al. 2016; Poretti 2016; Takahashi 2016). Briefly, mice were anesthetized with intraperitoneal injection of ketamine (75 mg/kg; Troy Laboratories Pty Ltd, USA) and medetomidine (1 mg/kg; Troy Laboratories Pty Ltd, USA). A midline incision was made in the skin overlying the skull, and the skin was retracted to reveal the bregma and skull overlying the anterior cranial fossa. Bilateral burr holes 2 mm in diameter were drilled in the skull 4 mm rostral and 2 mm lateral to bregma. The olfactory bulbs were aspirated, and small pieces of haemostatic sponge were used to stop the bleeding before the skin was sutured. Animals receiving a sham operation were treated identically, but the olfactory bulbs were left intact. Following surgery, animals were returned to their home cage. All animals had a 2-week recovery before starting fluoxetine hydrochloride or rTMS treatment. During the second week, all mice were

handled daily for 10 minutes to habituate the animals to the handling associated with rTMS treatment.

Repetitive Transcranial Magnetic Stimulation

rTMS was delivered daily on weekdays for 3 minutes, to deliver 1800 pulses per session, for 4 weeks (Figure 2.1 A-J). Detailed specifications of the custom coils and stimulators have been published previously (Makowiecki et al. 2014; Tang et al. 2016b) and



parameters are described briefly herein. For LI-rTMS, a custom-design copper coil (diameter, 8 mm) was connected to a modified E-cell (Global Energy Medicine, Australia). Magnetic field intensity at the coil base was approximately 12 mT (Makowiecki et al. 2014), and at the cortical surface was 4mT. For MI-rTMS, a custom-designed air core copper coil (diameter, 8 mm) was controlled by a waveform generator (335141B; Agilent Technologies, USA) connected to a bipolar programmable power supply (BOP 100-4M; Kepco Inc, USA). Magnetic field intensity at the coil base was approximately 90 mT (Tang et al. 2016b) and at the cortical surface was 50 mT. For HI-rTMS, a commercially available rat coil (Cool-40 Rat Coil; MagVenture A/S, Denmark) with a 40-mm diameter was used, powered by a MagPro R30 stimulator (MagVenture A/S, Denmark) set to 20% of capable output (Magnetic field intensity coil surface: 1.2 T; cortical surface: 1.0T). The peak magnetic field adjacent to the coil was measured with a Hall-effect probe (SS94A2D; Honeywell International Inc, USA). Sham-treated animals were handled as if receiving treatment, but the stimulator was not turned on. During treatment, the coil was placed in an anterior position between the ears, overlying the anterior cranial fossa to reliably target the prefrontal cortex bilaterally (Figure 1B, 1E, and 1H). Magnetic field measurements (in dB/dT and mT) for each coil are shown in figure 1. A discussion of the magnetic field intensities used in relation to human rTMS parameters is provided in supplemental information (Supplemental Text and Table 2.S1).

Fluoxetine Treatment

Fluoxetine (Sigma-Aldrich) 18 mg/kg was administered daily for 28 consecutive days, delivered in portioned balls of cookie dough (Corbett et al. 2012). Mice were monitored individually to ensure they had eaten the full quantity. Control mice (called vehicle group) received the same handling and were given a ball of cookie dough without fluoxetine.

Ingenuity Pathway Analysis

Metabolites identified by targeted metabolomics were uploaded into Ingenuity Pathway Analysis (IPA; Qiagen, Redwood City, CA) for stratification and categorization of direct and indirect network interactions using IPA's functional analysis algorithm and curated IPA Ingenuity Knowledge Base (IPAIB). IPA is a web-based software application that

allows for the analysis, integration, and interpretation of 'omics data sets by utilizing known molecular and genetic pathways and established relationships with cellular processes and metabolites. We utilized this integrative database in order to predict or identify pathways and processes affected by bulbectomy and multiple rTMS parameters. Prior to entry into IPA, each dataset of identified metabolites was sorted by CAS number. A metabolomics analysis was carried out using default IPA settings, excluding pathways specific to cancer cell lines. To minimize the incidence of false positive results, expression value threshold filters were set to a 1.25 fold-change ratio between bulbectomized and sham-operated animals with a minimum corrected confidence value of $p \leq 0.05$. The threshold filter for analysis of rTMS treatment was set at 1.5 fold-change ratio between treated and untreated groups. Using these criteria, IPA was able to generate a reference data set consisting of all significant and non-significant metabolites identified in sham treated animals and animals treated with various intensities of rTMS, as well as a focus set of metabolites consisting of those present in significantly different levels than the reference data set. Biological functions, disease states, and canonical pathways associated with our reference and focus set of metabolites were generated by IPA. The IPA functional analysis generated statistical significances derived from the association of our focus metabolite data sets with molecules already established with biological processes and canonical pathways using a right-tailed Fisher's Exact Test, where $p \leq 0.05$ was considered significant.

Data Analysis

Statistical analysis and graphing were carried out using GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA). Behavioural measures, brain 5HT and BDNF levels, and the number of proliferating cells were analysed using ANOVA or MANOVA with Sidak post hoc or Dunnett's T. For forced swim test data, we analysed treatment effects within-subjects, expressed as the difference between post-surgery and post-treatment scores. All data met the homogeneity of error variance assumption, except for BDNF frontal cortex data which were log transformed and then met assumptions.

The intensity-dependent effects of rTMS on plasma metabolites were analysed by one-way ANOVA with Tukey's post-hoc analysis. IPA was carried out by averaging the metabolite concentrations under each treatment parameter and comparing these mean values to the average metabolite concentration of the relevant control group in order

to calculate the fold-change in metabolite concentration. We also investigated possible correlations between FST performance and BDNF levels, neurogenesis and metabolite levels. The threshold for statistical significance for all experiments was set at $p < 0.05$ except where corrected p-values were used and these cases are indicated in the text.

Power calculations from the first set of behavioural (FST) data including MI-rTMS and fluoxetine demonstrated that $n=4$ (per group) would provide 0.8 power to detect a 15% differences ($p < 0.05$) in a 1-Way ANOVA with 4 Pairwise comparisons, with two-sided equality.

All animals underwent behavioural testing and were then randomly allocated to a particular type of post-mortem test. We assumed that the post-mortem tests would be less variable than behavioural tests, so animals were allocated to various tests to ensure that each group size was a minimum of $n=4$. The variation in group size was due to our efforts to reconcile randomizing animals from different surgery sessions, technical and timing requirements of experiments, a small number of experimental failures, and attempting to keep group size within experiments as similar as possible. Only one group (OB-Sham for 5HT ELISA) was smaller than 4, but that dataset had low variability, and a subsequent power analysis confirmed that the sample size provided 0.9 power.

Supplemental Information

The Supplemental Appendix describes an estimate of motor thresholds for each rTMS intensity, 5-ethynyl-2'-deoxyuridine (EdU; Thermo Fisher Scientific) labelling of newly born cells, behavioural analysis, and brain and serum collection. The Supplemental Appendix also summarizes serotonin and BDNF enzyme-linked immunosorbent assay, analysis of neurogenesis, and metabolomics.

Results

Tolerability

rTMS at all intensities was well tolerated by the mice and there was no sign that the animals felt any sensations during the stimulation and no seizures, freezing or avoidance behaviours were noted for any of the intensities applied. Furthermore, there were no signs of ongoing pain or discomfort following the stimulation as determined by the facial grimace score (Langford 2010).

Reduction of Hyperactivity in the FST by MI-rTMS But Not Fluoxetine Is Independent of Serotonin Levels

As previously reported (Linge, Pazos & Diaz 2013; Mucignat-Caretta, Bondi & Caretta 2004; Mucignat-Caretta, Bondi & Caretta 2006), the FST revealed a significant and reliable decrease in immobility after OB surgery, providing an opportunity to compare the efficacy of rTMS and fluoxetine in restoring normal activity levels. Activity in the FST was not significantly different between groups at baseline (pre-intervention; figure 2.2A) but significantly differed in the change induced by treatment over time (analysis of variance [ANOVA], significant time \times group interaction with Greenhouse-Geisser correction for sphericity violation, $F(7.06, 164.91)=3.702$ ($P=0.001$). Follow-up tests for simple effects of time within each group showed a significant decrease in immobility time in the FST from presurgery to postsurgery in all groups (P values $<.05$) except for sham/sham ($P=0.42$), confirming that OB induced hyperactivity consistent with a phenotype of agitated depression (Figure 2.2B).

To compare the efficacy of MI-rTMS and fluoxetine in reducing OB-induced hyperactivity, we performed further follow-up analyses (Dunnett test), examining rTMS and fluoxetine groups compared with their respective controls and with sham/sham. The MI-rTMS group showed significantly greater improvements between the postsurgery to posttreatment time points in the FST than both OB/sham (Dunnett test, $P=0.03$) and sham/sham (Dunnett test, $P=0.02$) (Figure 2.2B). However, no significant difference was found between postsurgery and posttreatment for fluoxetine or vehicle groups (Dunnett test, $P>.05$) (Figure 2.2B). We then investigated whether the behavioural changes were accompanied by a change in 5-hydroxytryptamine (5HT) levels in the prefrontal cortex at the end of treatment. A significant difference was observed in prefrontal cortex 5HT levels between groups (ANOVA, $F[4, 38]=4.113$; $P=0.007$) (Figure 2.2C). Post hoc tests showed that MI-rTMS did not significantly affect 5HT, but the OB/fluoxetine group had significantly increased frontal cortex 5HT levels compared with both vehicle-treated control (Sidak, $P=0.03$) and sham/sham ($P=0.004$) (Figure 2.2C). Interestingly, the 2 control groups (OB/vehicle and sham/sham) were not significantly different from each other, suggesting that OB itself did not alter baseline 5HT concentration ($P=0.86$).

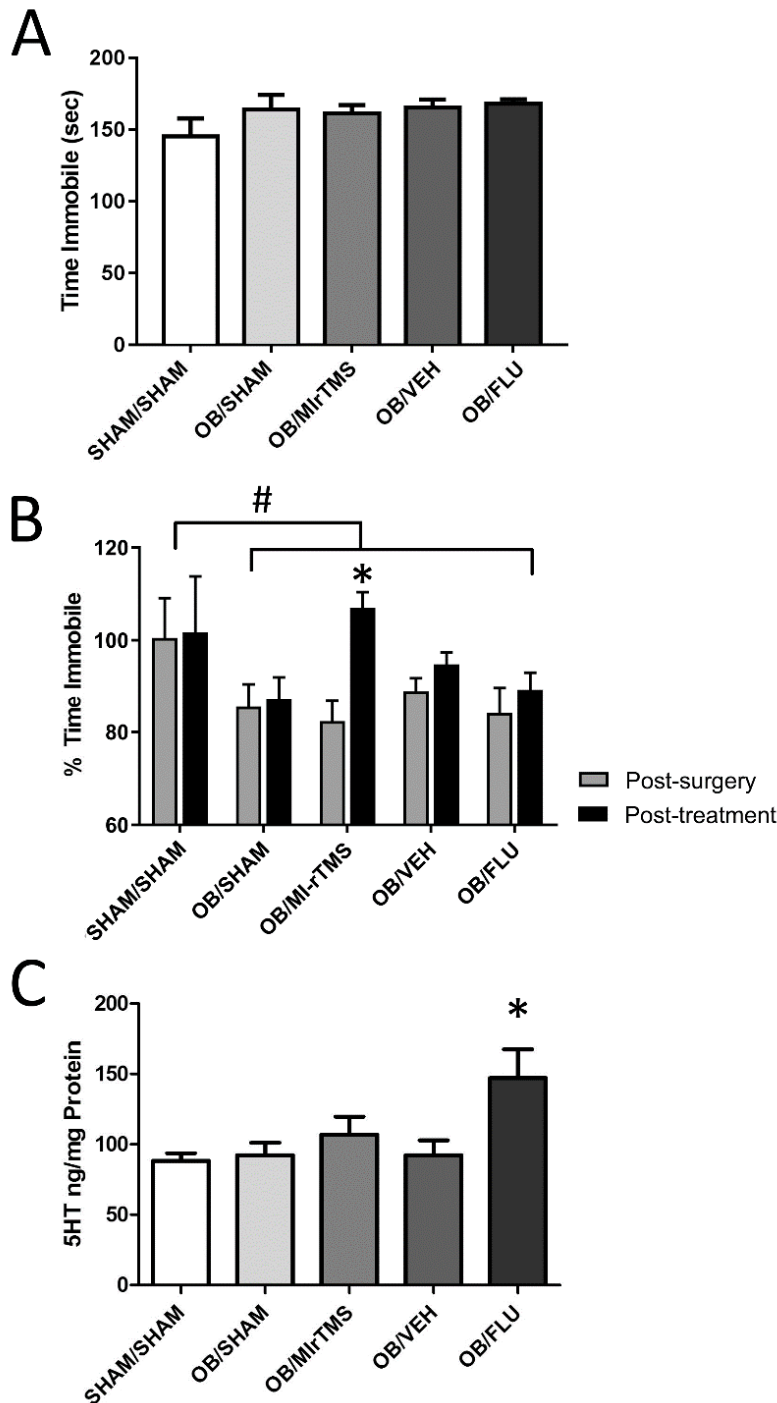


Figure 2.2 MI-rTMS and Flu Effects in Mice with OB. A, Time (in seconds) spent immobile at baseline (pre-surgery) for all groups, confirming that there were no differences between animals at the start of the experiment. B, Time spent immobile, expressed as a percentage of presurgery values of mice with intact olfactory bulbs (sham/sham) and mice with OB treated with MI-rTMS or Flu. For all groups, OB resulted in a significant decrease in the time spent immobile, representing hyperactivity. Behaviour was significantly improved by 4 weeks of treatment with MI-rTMS but not Flu. # indicates that postsurgery levels are significantly different than for untreated animals. * indicates significant differences between postsurgery and posttreatment within a group. C, Concentration of 5HT in the frontal cortex of sham/sham mice and mice following OB surgery treated with MI-rTMS or Flu. OB alone had no effect on 5HT levels. * indicates that only Flu increased 5HT levels following the OB procedure. Error bars represent standard error of the mean. 5HT indicates 5-hydroxytryptamine; Flu, fluoxetine hydrochloride; MI-rTMS, medium-intensity repetitive transcranial magnetic stimulation; OB, olfactory bulbectomy; Veh, vehicle (i.e., control).

Intensity of rTMS Differentially Reduces Hyperactivity in the FST

We then delivered rTMS at different intensities to determine optimal conditions for reducing hyperactive behaviour in the FST. We first confirmed that there were no pre-existing differences in the FST behaviour prior to intervention (Figure 2.3A). A significant change was observed in immobility time in the FST over time and across groups (ANOVA, $F[2, 144]= 54.64; P<.001$), indicating that surgery increased activity (agitation) in the FST. All rTMS groups showed some recovery, but only the MI-rTMS ($P=0.005$) and HI-rTMS ($P=0.02$) groups improved significantly between postsurgery and posttreatment (Figure 2.3B). Confirming our results from the first experiment, none of the rTMS groups showed any change in 5HT levels in the frontal cortex (ANOVA with groups OB-sham, LI-rTMS, MI-rTMS, and HI-rTMS ($F[3, 25]=1.106; P=0.37$) (data not shown).

Brain-Derived Neurotrophic Factor

Our initial experiment ruled out a role for 5HT in the effects of MI-rTMS and in the rescue of OB-induced behaviour. Therefore, we investigated prefrontal cortical and hippocampal BDNF levels, as well as hippocampal neurogenesis, as possible mechanisms underlying behavioural change in the cohorts of different rTMS intensity.

ANOVA showed a significant main effect of rTMS treatment on BDNF concentrations in the frontal cortex (ANOVA, $F[4, 29]=3.368; P=0.02$) and the hippocampus ($F[4, 29]=2.983; P=0.04$). We followed up by first comparing all groups to the sham/sham group. In the frontal cortex, only the MI-rTMS group had significantly higher BDNF levels than the sham/sham group (Dunnett test, $P=0.048$) (Figure 2.3C). In the hippocampus, BDNF levels were increased to a similar level in the MI-rTMS, HI-rTMS, and OB-sham groups compared with the sham/sham group, although this increase was significant only for MI-rTMS (Dunnett test, $P=0.04$) (Figure 3D). No significant differences in BDNF levels were observed between any rTMS-treated group and the OB-sham group (Dunnett test, $P>.05$).

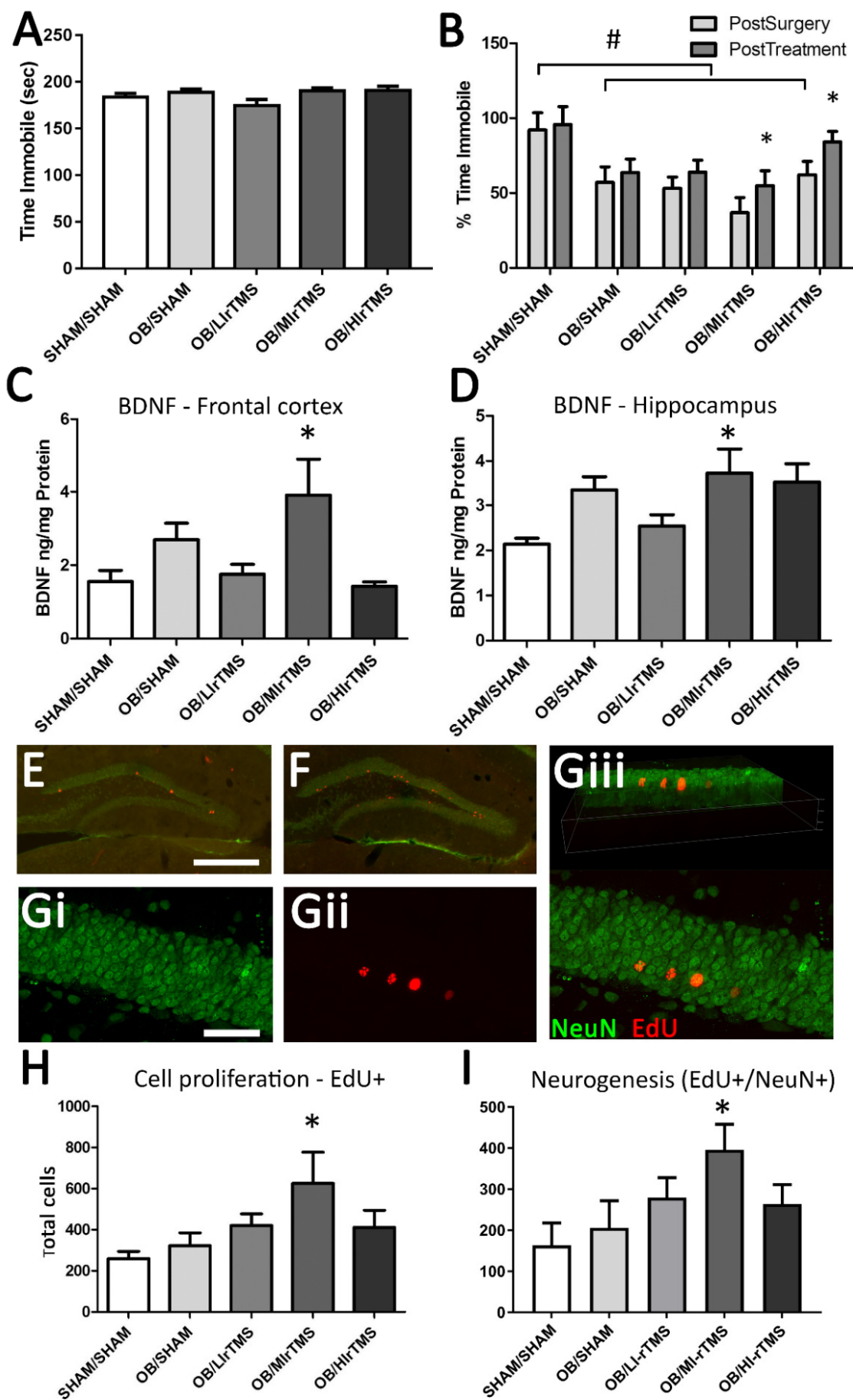


Figure 2.3 Comparison of rTMS Intensities. A, Time (in seconds) spent immobile at baseline (pre-surgery) for all groups, confirming that there were no differences between animals) at the (continued on next page)

start of the experiment. B, Time spent immobile in the forced swim test as a percentage of the presurgery value for each animal. OB surgery significantly reduced the time spent immobile compared with presurgery values, indicating hyperactivity. Behaviour was partially rescued by MI-rTMS treatment. # indicates that postsurgery levels are significantly different from untreated animals. * shows significant differences between postsurgery and posttreatment within a group. C,D, BDNF concentration in the frontal cortex (C) and hippocampus (D). Only MI-rTMS resulted in increased BDNF levels compared with sham/sham treatment in both frontal cortex and hippocampus, but no significant differences were observed between any groups compared with OB/sham. * indicates a significant difference compared with sham/sham. E,F,G, Immunohistochemistry showing EdU single-labelled and EdU-NeuN double-labelled cells in the granular and subgranular layers of the dentate gyrus. E,F: low power view of the hippocampus in sham (E) and MI-rTMS (F) treated mice showing increased Edu labelling following MI-rTMS. I, confocal microscopy of MI-rTMS treated animal confirming co-localization of NeuN (Gi), EdU (Gii), labels merged (Giii), with a 3d rotated view showing colocalization of red and green staining within the hippocampal neurons (Giii). H,I, Number of EdU-positive cells (H) and Edu-NeuN double-labelled cells (I) in hippocampus following OB surgery and rTMS treatment. A significant increase was found in the number of newly born neurons following MI-rTMS compared with OB/sham and sham/sham mice. * shows a significant difference compared with sham/sham and OB/sham. Error bars represent standard error of the mean. Scale bars are 500 μm (E) and 50 μm (G). BDNF indicates brain-derived neurotrophic factor; EdU, 5-ethynyl-2'-deoxyuridine (Thermo Fisher Scientific); HI-rTMS, high-intensity repetitive transcranial magnetic stimulation; LI-rTMS, light-intensity repetitive transcranial magnetic stimulation; MI-rTMS, medium-intensity repetitive transcranial magnetic stimulation; NeuN, neuron-specific protein; OB, olfactory bulbectomy; rTMS, repetitive transcranial magnetic stimulation.

Hippocampal Neurogenesis

In a separate cohort, we analysed cell genesis by injecting EdU at the midpoint of rTMS treatment. The mice were killed humanely 2 weeks later. We compared the density of newly born neurons (EdU and neuron-specific protein [NeuN] double-labelled cells) following the different rTMS intensities (Figure 2.3E-G). A significant difference was detected between groups (ANOVA, $F[1, 24]=2.989$; $P=0.04$), but only MI-rTMS had a significantly higher number of newly born neurons (Figure 2.3E,F,H, I) compared with the sham/sham group (Dunnett test, $P=0.02$). Further follow-up analyses showed that hippocampal neurogenesis in MI-rTMS was also significantly greater than OB-sham (Dunnett test, $P=0.02$), but no other groups were significantly different to the OB-sham group (Dunnett test, $P>.05$) (Figure 2.3E,F,H,I). The percentage of newly born neurons positive for NeuN was not significantly different between groups (data not shown).

Plasma Metabolomics

The metabolic effects of OB and the different intensities of rTMS treatment were examined using targeted metabolomics driven by ultrahigh performance liquid chromatography. In total, 6 of the 42 examined metabolites were found to be significantly altered in the plasma of OB mice compared with their sham-treated counterparts. OB decreased the concentration of 3 bioactive amines— γ -amino-butyric acid (GABA) ($P=0.049$), AABA ($P=0.001$), and their molecular precursor glutamine

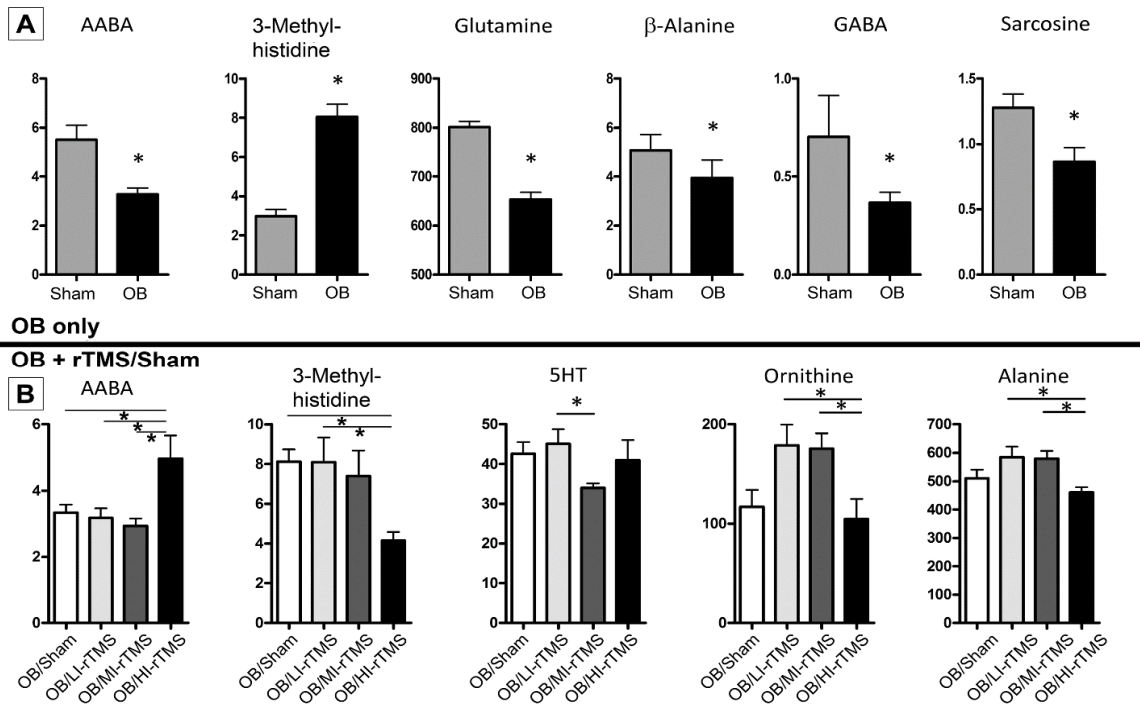


Figure 2.4 Metabolomics of OB Surgery and rTMS Exposure. A, The OB surgery decreased AABA, glutamine, β -alanine, GABA, and sarcosine levels. OB surgery increased 3-methylhistidine. B, After the OB procedure, rTMS exposure appeared to interact with 7 metabolites, but post hoc analyses showed that only AABA was significantly upregulated and 3-methylhistidine was significantly downregulated with HI-rTMS. However, 5 metabolites—AABA, 3-methylhistidine, 5HT, ornithine, and alanine—were differentially affected by different stimulation intensities. Units are μ M. AABA indicates α -aminobutyric acid; 5HT, 5-hydrotryptophan; GABA, γ -aminobutyric acid; HI-rTMS, high-intensity repetitive transcranial magnetic stimulation; LI-rTMS, low-intensity repetitive transcranial magnetic stimulation; MI-rTMS, medium-intensity repetitive transcranial magnetic stimulation; OB, olfactory bulbectomy; rTMS, repetitive transcranial magnetic stimulation.

($P < .001$)—as well as β -alanine ($P = 0.048$) and sarcosine ($P = 0.04$). Only 3-methylhistidine was significantly increased by OB ($P < .001$) (Figure 2.4A). After the OB procedure and rTMS treatment at different intensities, 7 of 42 examined metabolites were determined to significantly interact with rTMS (Supplemental Table 2.S2). However, post hoc analysis revealed that only AABA and 3-methylhistidine were affected by any specific rTMS treatment, being significantly upregulated ($P = 0.004$) and downregulated, respectively, by HI-rTMS ($P = 0.03$) (Figure 2.4B). Interestingly, these 2 metabolites were also significantly altered by OB, and HI-rTMS had the effect of restoring normal levels. All rTMS intensities produced different changes in the plasma metabolic profile, and post hoc analysis revealed that 5 of the 7 metabolites were affected differentially by different stimulation intensities (Figure 2.4B).

IPA revealed that the top 4 canonical pathways affected by OB included glutamate-dependent acid resistance ($P = 0.006$), glutamine degradation ($P = 0.009$), glutamine

biosynthesis ($P=0.02$), and GABA receptor signalling ($P=0.02$). Glutamate receptor signalling was also significantly affected by OB ($P=0.02$) (Supplemental Figure 2.S1).

The IPA results were suggestive that various rTMS stimulation parameters had differential effects on peripheral metabolites. For example, LI-rTMS altered metabolites associated with glutamate-dependent acid resistance ($P=0.006$), inducible nitric oxide synthase signalling ($P=0.009$), the antiproliferative role of the somatostatin II receptor ($P=0.01$), inhibition of angiogenesis by thrombospondin-1 ($P=0.01$), and neuronal nitric oxide synthase signalling in neurons ($P=0.01$) (Supplemental Figure 2.S2A). MI-rTMS significantly affected some of the same canonical pathways, such as glutamate-dependent acid resistance ($P<.001$) and neuronal nitric oxide synthase signalling in neurons ($P<.001$), but also affected arginine biosynthesis ($P<.001$), the citrulline metabolism superpathway ($P<.001$), and aspartate biosynthesis ($P<.001$) (Supplemental Figure 2.S2B). LI-rTMS and MI-rTMS also modulated canonical pathways associated with glutamate degradation and GABA receptor signalling (Supplemental Figure 2.S2A and 2.S2B). Similar to other rTMS stimulation paradigms, HI-rTMS significantly altered glutamate-dependent acid resistance ($P=0.006$) and GABA receptor signalling ($P=0.02$) (Supplemental Figure 2.S2C). It also upregulated canonical pathways involved in glutamate degradation ($P=0.02$), albeit by altering a different branch of the catabolic pathway. HI-rTMS also significantly altered canonical pathways associated with histamine biosynthesis ($P=0.006$) and ceramide degradation ($P=0.02$). Notably, glutamate-dependent acid resistance, glutamate degradation, and GABA receptor signalling pathways were downregulated by OB and upregulated by all rTMS intensities.

Correlations

When all animals were included in the analysis, FST performance post treatment showed a significant negative correlation with BDNF levels (Frontal cortex: Pearson's correlation -0.365 ; $P=0.034$; Hippocampus: Pearson's correlation -0.359 ; $P=0.037$) and a significant positive correlation AABA (Pearson Correlation $.474$; $P=0.029$), but these correlations were not observed within any individual treatment group. When groups were analysed separately, only animals in the Sham-OB group showed a positive correlation with 3-methyl histidine (Pearson's correlation 0.694 ; $P=0.038$).

Discussion

To our knowledge, this is the first study to suggest that MI-rTMS and HI-rTMS improved hyperactivity in mice with OB, while fluoxetine did not. These findings suggest that OB may be a valid murine model of an agitated, treatment-resistant depression. MI-rTMS-treated mice had increased BDNF levels in the frontal cortex and hippocampus, as well as increased neurogenesis in the hippocampus. None of the rTMS-treated groups had increased serotonin levels. Plasma metabolites such as AABA and 3-methylhistidine, which are involved in glutamate and GABA metabolic signalling pathways, may have utility for further translational research of biomarkers for rTMS.

Similar to the OB model used in the present study, some forms of depression in humans are associated with an agitated phenotype marked by increased stress reactivity and psychomotor agitation, increased nocturnal activity, and impaired concentration (Lin, Huang & Liu 2016). Previous studies of OB in mice have consistently revealed a phenotype of psychomotor agitation (Linge, Pazos & Diaz 2013; Mucignat-Caretta, Bondi & Caretta 2004; Mucignat-Caretta, Bondi & Caretta 2006) and we show for the first time that this behaviour is resistant to treatment with fluoxetine, similar to clinical human populations with treatment-resistant depression (Gili 2012; Lin, Huang & Liu 2016; McGrath 2008; Parker 1999; Su 2017). Previous studies have demonstrated that development of the agitated phenotype is influenced by strain and the time postsurgery (Otmakhova et al. 1992; Roche et al. 2012). Future efforts should include a comprehensive battery of behavioural tests examining locomotion, activity, and anxiety, as well as circadian rhythm and hedonic behaviours, in response to OB and antidepressant treatment, to further characterize phenotype and validity of the model.

Although many studies have investigated the role of rTMS frequency on brain excitability and function, less focus has been given to the role of intensity, perhaps because the mechanisms activated by rTMS have always been assumed to involve traditional forms of synaptic plasticity such as long-term potentiation and long-term depression, which require action potential firing (Thickbroom 2007). However, recent work in humans and in animal models has shown that LI-rTMS and MI-rTMS (subthreshold) can have a notable effect on the brain (Boggio 2010; Cirillo 2017; Clarke et al. 2017). In our study, both MI-rTMS and HI-rTMS reduced FST hyperactivity in the OB model despite being sub- and at-threshold respectively, and causing different neurobiologic outcomes and

recruiting different mechanisms of action, as described. These results contrast with previous studies showing that rTMS induced hyperactivity in the open field in intact, normal rats (El Arfani 2017) although extensive differences in stimulation parameters, coil geometry, model and species preclude a useful comparison between these two studies. Nonetheless, reports of rTMS modulating activity in preclinical studies suggest that it will be important to elucidate whether the improvement in FST following MI and HI-rTMS was due to rescue of the OB-induced psychomotor agitation, or a more general effect due to increased psychomotor inhibition.

Our experiments do not allow us to determine which of MI or HI-intensity provides better outcomes in our model because the behavioural response was similar for both intensities. However, we speculate that MI-rTMS may provide longer lasting anti-depressant effects than HI-rTMS due to the increase in neurogenesis observed only at medium intensity. The lack of effect on neurogenesis of HI-rTMS suggests a transient anti-depressant effect, and is consistent with the occurrence of relapse in human patients once treatment has ceased. It will be important to carry out longitudinal studies to determine when anti-depressant effects are first observed and how long they persist. It would also be interesting to test a 2-step treatment protocol: initial delivery of HI-rTMS might provide rapid benefits, which could then be followed with MI-rTMS to integrate these benefits into long term structural changes through increases in neurogenesis.

Our work confirms previous suggestions that MI-rTMS upregulates BDNF following OB, in various limbic structures including the frontal cortex and hippocampus (Freitas et al. 2013; Hellweg et al. 2007), but the involvement of the protein in the behavioural phenotype is still unknown, just as the relationship of BDNF to depression in humans is still under debate (Duman et al. 2016; Duman et al. 1997; Hellweg et al. 2007; Kheirouri et al. 2016). The present data argue against the possibility that increased BDNF level could be a simple consequence of increased locomotor activity following OB (Engesser-Cesar et al. 2007; Ferris et al. 2007), because MI-rTMS reduced hyperactivity while increasing BDNF concentration. Rather, BDNF upregulation may be a beneficial response to MI-rTMS with increased neuroplasticity (Freitas et al. 2013). The increase in BDNF induced by MI-rTMS may have been sufficient to drive neurogenesis in the hippocampus: Likely, BDNF levels and neurogenesis are linked in our study because

hippocampal BDNF levels have been causally linked with neurogenesis in mouse models of learning, enrichment, and anxiety (Begni et al. 2017; Lim 2016; Sleiman 2016; Zhang et al. 2015b). Interestingly, MI-rTMS increased the number of newly generated cells detected 3 weeks after the EdU injection but did not selectively increase the proportion of these cells that differentiated into neurons. Future work could examine whether MI-rTMS increases proliferation or simply increases the survival of newly born cells, because this result has implications for understanding the integration of newly born cells into the hippocampal network (Abrous & Wojtowicz 2015; Kempermann 2015). It will also be interesting to investigate possible cognitive changes following OB and MI-rTMS treatment, because increased neurogenesis is associated with changes in learning and memory (Snyder et al. 2005b).

Our negative neurobiologic outcomes for HI-rTMS contrast with previous studies that showed increases in both BDNF (Gersner et al. 2011; Muller et al. 2000) and neurogenesis (Ueyama et al. 2011) following rTMS using a human coil. The different cellular responses to stimulation at similar intensities may be due to differences in the focality of the coils. We used a commercial rat coil (MagVenture) that not only delivers a broader field than our small animal coils (Figure 2.1) but also differs in shape from the conventional human coils (such as figure 8 coils or large circular coils) used in previous studies (Grehl et al. 2015). We also did not observe an increase in brain BDNF levels following LI-rTMS, consistent with previous work showing that very low-intensity stimulation may be brain region specific: LI-rTMS upregulates BDNF in the visual cortex, midbrain, and cerebellum but not in the retina (Morellini 2015; Rodger et al. 2012; Tang et al. 2015), and the lack of upregulation of BDNF in the hippocampus is consistent with our previous study showing no change in hippocampal dendritic spines following LI-rTMS (Sykes et al. 2013). Nonetheless, we cannot rule out a localized upregulation in the dentate gyrus with these intensities, which was not detected in our dissection of the whole hippocampus. Overall, our findings highlight that the range of mechanisms called into play by different rTMS intensities has implications for outcomes in different brain regions that may receive different field strengths simply as a result of being at a distance from the focal point of stimulation (Deng, Lisanby & Peterchev 2013).

Our serum analysis identified several metabolites that were altered in response to OB and in response to 1 or more rTMS frequencies. However, the concentrations of only 2

metabolites, AABA and 3-methylhistidine, were increased and decreased, respectively, by OB and restored to baseline levels by HI-rTMS treatment but not by LI-rTMS or MI-rTMS. This finding further highlights that MI-rTMS and HI-rTMS have specific effects on the different brain and peripheral markers of agitated depression modelled in our study, even though they may eventuate in similar behavioural outcomes. Interestingly, AABA has been identified as a potential biomarker for treatment-resistant depression in humans (Woo 2015), consistent with the relatively high efficacy of rTMS in treating these patients (Brunoni 2017; Fitzgerald 2006; George 2010). Therefore, evidence from animal and human studies suggests that AABA and 3-methylhistidine may serve as biomarkers in humans for phenotyping depression. Further longitudinal work could determine if AABA and 3-methylhistidine have utility for monitoring treatment response to rTMS or precision medicine approaches to brain stimulation interventions (Brunoni 2017; Fitzgerald 2006; George 2010).

Our IPA identified glutamate degradation, glutamate-dependent acid resistance, GABA receptor signalling, and 4-aminobutyrate degradation as systems that were both downregulated following OB surgery and upregulated by HI-rTMS and, to a lesser extent, MI-rTMS. Consistent with our findings, changes in GABAergic and glutamatergic processes in the brain have been reported following OB (Beauchemin et al. 1994; Dennis et al. 1993; Ho et al. 2000; Jancsar & Leonard 1984) and in the brain and blood of depressed patients (Altamura et al. 1993). Furthermore, rTMS in rodents and in humans can selectively alter GABA and glutamate levels in the brain and blood (Dubin et al. 2016; Enzi 2012; Yue, Xiao-lin & Tao 2009). This result adds to clinical evidence that behaviours associated with depression may be mediated by GABAergic and glutamatergic systems (Hasler & Northoff 2011; Lener 2017; Luscher et al. 2011; Murrough et al. 2017; Yue, Xiao-lin & Tao 2009).

The present findings and prior work suggest that significant changes occur in glutamate and other amino acids in the blood of depressed patients (Murrough, Abdallah & Mathew 2017). However, the implications of this outcome are uncertain because peripheral measures do not necessarily reflect brain activity (Palsson 2015; Setoyama 2016). A possible explanation is that activation of the hypothalamic-pituitary-adrenal axis and the autonomic nervous system impacts peripheral metabolite levels in the blood (Arakawa et al. 1997; Lamers 2013; Musselman et al. 1998; Sachar 1973). For

example, in rats, activation of the sympathetic nervous system increases the activity of glutamate pyruvate transaminase and glutamateoxaloacetate transaminase in the blood. These enzymes accelerate the conversion of glutamate to 2-ketoglutarate, resulting in a net decrease in plasma glutamate levels. Prior work demonstrates that corticotrophin-releasing hormone and adrenaline reduce blood glutamate concentrations in rats (Zlotnik 2010). This explanation is compatible with our pathway analysis, implicating glutamate and GABA pathways in OB and in MI-rTMS and HI-rTMS-induced recovery.

In summary, our work suggests that the OB murine model may have utility as a model for agitative, treatment-resistant depression. Future studies with exhaustive behavioural testing could assist with the validation of this model. Variations in rTMS intensity appear to alter brain and metabolomic effects in the OB mouse model of agitated depression. Further work in this area could assist with the optimization of clinical rTMS delivery. Peripheral metabolomic markers might have utility as biomarkers for the diagnosis of specific forms of depression and in monitoring or modifying rTMS treatment. Further translational studies are warranted.

Supplemental Methods and Results

Relationship of intensities used in the current study to rTMS intensities used in humans: estimation of motor threshold vs raw magnetic field intensities.

A key question is how the intensities of rTMS applied here in mice can be appropriately translated to humans. Although motor evoked potentials (MEP) are the most commonly used tool to estimate intensities in human treatment, we do not think that MEPs are a useful measure when comparing mouse and humans due to differences in coil parameters.

In order to obtain MEP data from mice, it is necessary to use large coils that deliver high intensity fields (1-2 Tesla) to depolarize cortical neurons. Our small coils are simply not strong enough to elicit a MEP, the current required to induce such high intensity fields would melt the coil. Although it provides a strong enough field, the commercial “rat” coil, does not elicit reliable MEPs even in rats¹ presumably due to the shape of the coil.

When a large coil is used, these coils tend to be larger than the size of the mouse head, resulting in low magnetic induction efficiency. As a result, the intensity used to elicit a MEP in a mouse is not a true reflection of the intensity required to activate the motor cortex. At the moment, it is not possible to establish a direct comparison between mouse and human MEPs due to the technical limitations imposed by the coils.

Nonetheless, we attempted to make some rough comparisons as follows: using the commercial rat coil on an anaesthetized mouse, 43% maximum stimulator output (MSO) was required to elicit a visible twitch in the mouse forelimb with every stimulation. We reduced the MSO until the twitch just disappeared, avoiding any potential discomfort or sensation to the awake mice in our experiments, which would have confounded our analysis of depression-like behaviours. The MSO was 20% and we calculated the field intensity at the surface of the coil to be 1.2T, and the field intensity at the surface of the cortex to be 1.0T. We called this amount the motor threshold in mice, and have related all other intensities to this value, regardless of coil shape or size.

In translating our findings to humans, we hypothesise that the intensity required to depolarize a mouse neuron will be similar to the intensity required to depolarize a human neuron. We therefore suggest that due to differences in coil size and shape, the best approach is to match the magnetic field intensity, as well as the induced electric

field intensity and distribution, at the surface of the cortex in both mice and humans. Our approach also has the advantage that we can take into account the distance between the coil surface and the cortical surface, which is 10 times greater in humans than in mice (Supplemental Table 2.S1).

Table 1.S1 Animal and Human Coil Comparisons

Coil	Intensity at Coil Surface (mT)	Intensity at Cortical Surface (2 mm distance from base of coil) mT	% MSO (Magventure rat coil only)	% MT (estimated)
HI-rTMS coil (Rat coil)	1200	1000 (37,312)	20	100
MI-rTMS coil	90	51 (128)	NA	5
LI-rTMS coil	12	4 (14)	NA	0.4

HI-rTMS, high-intensity repetitive transcranial magnetic stimulation; LI-rTMS, low-intensity repetitive transcranial magnetic stimulation; MI-rTMS, medium-intensity repetitive transcranial magnetic stimulation; mm, Millimeter; mT, Millitesla ; %MSO, percentage of maximum stimulator output; %MT, percentage of motor threshold.

Edu labelling of newly born cells

At the start of the third week of treatment, 50% of mice in each group were randomly chosen to receive an injection of EdU (50mg/kg) to label dividing cells. The remaining 50% were used for analysis of brain metabolites (see below). Two cohorts were necessary as it was not possible to collect frozen and perfused tissue from the same animals.

Behavioural Analysis

For the forced swim test, mice were placed in a 2,000 mL glass beaker half-filled with $25 \pm 1^\circ\text{C}$ tap water for 6 minutes and were monitored with video recording. The latency to the first immobility was recorded, and the total immobility time during the last 4 minutes was measured by a blinded research assistant. Immobility was defined by lack of translational movement and a paucity of limb and body movements beyond those necessary to maintain balance and buoyancy. Mice were removed from the water and thoroughly dried on a heating pad prior to return to their homecage.

Brain and serum collection

Immediately, following the final behavioural test, mice were injected with 0.1ml lethobarb (325mg/ml pentobarbital, Virbac) and 500µl of blood was collected into capillary tubes by cardiac puncture and centrifuged at 10,000 g for 5 minutes to isolate an acellular plasma fraction. Mice were then processed either for ELISA assays (frozen tissue) or EdU immunohistochemistry (perfusions).

Serotonin and BDNF ELISA

For ELISA assays, the left frontal cortex and hippocampus were homogenized in buffer containing 100 mM PIPES H 7, 500 mM NaCl, 0.2% Triton X-100, 2mM EDTA and mini protease inhibitor tablets (Roche Biochemicals, Indiana USA; 1 per 50ml buffer; (Szapacs et al. 2004). Samples were centrifuged at 4°C for 1 hour and supernatants stored at -80°C. The total amount of serotonin in the frontal cortex was analysed using a Serotonin EIA kit (Enzo Life Sciences®). Both the frontal cortex and hippocampus homogenates were analysed using ChemiKine BDNF Sandwich Elisa Kit (Millipore). The total amount of protein in each sample was quantified with a BCA protein assay kit (Pierce® BCA Protein Assay Kit, Thermo Fisher Scientific©, Illinois USA). All plates were analysed using a PerkinElmer 2300 spectrophotometer with EnSpire software. A standard curve was plotted and fitted with a 4 parameter logistic non-linear curve using the Enspire software. Serotonin and BDNF concentrations (ng/mL) of samples were derived from the standard curve and normalised to the protein concentrations (ng/ml) calculated from each sample.

Analysis of Neurogenesis

Mice were transcardially perfused with 50ml of 4% paraformaldehyde in 0.1 Phosphate Buffer. Brains were postfixed in 4% paraformaldehyde solution at 4°C and transferred to 30% sucrose in PBS 48 hours before cryosectioning into 40µm sections. To identify neurons that had been newly generated during rTMS treatment, sections were stained with mouse anti NeuN (Merck Millipore) and then processed for EdU detection. Sections were blocked and permeabilised with 10% normal donkey serum in 0.1% Triton in 1M PBS then incubated overnight with the anti NeuN (1:400). The following day the sections were incubated with the secondary antibody Donkey anti Mouse (1:600; Life Technologies) and the nuclear dye Hoechst (1:1000; Sigma). Immediately after

immunohistochemistry was completed, sections were permeabilised in 0.5% Triton in PBS and washed in 3% BSA in PBS. Sections were incubated in the Click-it EdU reaction cocktail (AF594) for 30 minutes, washed in 3% BSA in PBS. Sections were mounted onto supafrost slides (Menzel Gläser, Lomb Scientific) and cover slipped with Fluoromount (Southern Biotech). Sections were analysed under a Leitz Diaplan fluorescent microscope (Leitz, Wetzlar, Germany) at 100x magnification. All EdU single labelled and EdU-NeuN double-labelled cells in the granular and sub granular layers of the dentate gyrus were counted. In each section, counts were made in either the left or right hippocampus based on random sampling. Twelve sections were counted in total per animal. For confocal imaging, we used a Nikon confocal C2, NI-E microscope with a 60x oil immersion lens. Sections were imaged using 36 Z-stacks at 1µm thickness and images were collected and maximum projections made using Nis Elements AR software.

Serum analysis: Metabolomics

Amino acids and their metabolites were measured by LCMS as previously described.^{3,4} Briefly, plasma samples were spiked with internal standards then deproteinized with cold methanol, followed by centrifugation at 10,000 g for 5 minutes. The supernatant was immediately derivatized with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate according to the manufacturer instructions of Waters' MassTrak kit. A 10-point calibration standard curve underwent a similar derivatization procedure after the addition of internal standards. Both derivatized standards and samples were analyzed on a triple quadrupole mass spectrometer coupled with an Acquity Ultra Pressure Liquid Chromatography (UPLC) system. Data acquisition was done using a select ion monitor (SRM). Concentrations of 42 analytes of each unknown were calculated against each perspective calibration curve.

Ingenuity Pathway Analysis

Metabolites identified by targeted metabolomics were uploaded into Ingenuity Pathway Analysis (IPA; Qiagen, Redwood City, CA) for stratification and categorization of direct and indirect network interactions using IPA's functional analysis algorithm and curated IPA Ingenuity Knowledge Base (IPA KB). IPA is a web-based software application that allows for the analysis, integration, and interpretation of 'omics data sets by utilizing known molecular and genetic pathways and established relationships with cellular processes and metabolites. We utilized this integrative database in order to predict or

identify pathways and processes affected by bullectomy and multiple rTMS parameters. Prior to entry into IPA, each dataset of identified metabolites was sorted by CAS number. A metabolomics analysis was carried out using default IPA settings, excluding pathways specific to cancer cell lines. To minimize the incidence of false positive results, expression value threshold filters were set to a 1.25 fold-change ratio between bullectomized and sham-operated animals with a minimum corrected confidence value of $p \leq 0.05$. The threshold filter for analysis of rTMS treatment was set at 1.5 fold-change ratio between treated and untreated groups. Using these criteria, IPA was able to generate a reference data set consisting of all significant and non-significant metabolites identified in sham treated animals and animals treated with various intensities of rTMS, as well as a focus set of metabolites consisting of those present in significantly different levels than the reference data set. Biological functions, disease states, and canonical pathways associated with our reference and focus set of metabolites were generated by IPA. The IPA functional analysis generated statistical significances derived from the association of our focus metabolite data sets with molecules already established with biological processes and canonical pathways using a right-tailed Fisher's Exact Test, where $p \leq 0.05$ was considered significant (Supplemental Figures 2.1S and 2.2S and Supplemental Table 2.2S).

Table 2.S2. Plasma Concentrations of Targeted Metabolomics Among the 4 Group Comparisons

Metabolite ^a	Plasma Concentration, Mean (SD), μM				rTMS Effect?	P Value
	OB/Sham	OB/LI-rTMS	OB/MI-rTMS	OB/HI-rTMS		
Histidine	62.79 (7.83)	79.92 (10.50)	83.90 (10.14)	88.97 (10.81)	No	.33
Hydroxyproline	16.68 (1.83)	16.72 (1.08)	16.81 (1.35)	15.75 (0.88)	No	.94
1-Methylhistidine	2.80 (0.51)	3.55 (0.64)	4.01 (0.67)	2.67 (0.65)	No	.34
3-Methylhistidine	8.10 (0.63)	8.04 (1.23)	7.41 (1.29)	4.03 (0.43)	Yes	.03
Asparagine	34.98 (1.99)	41.31 (4.18)	41.93 (3.93)	42.57 (3.51)	No	.46
Phosphoethanolamine	3.26 (0.71)	2.07 (0.51)	3.19 (0.77)	4.68 (1.05)	No	.13
Arginine	78.89 (14.37)	45.57 (14.18)	49.31 (16.59)	101.50 (13.52)	Yes	.04
Carnosine	3.85 (0.70)	3.70 (0.45)	5.32 (1.56)	4.19 (0.82)	No	.63
Taurine	770.80 (67.50)	755.20 (40.55)	944.0 (104.30)	729.70 (75.76)	No	.18
Anserine	6.43 (1.31)	5.97 (1.02)	8.95 (2.79)	6.10 (1.57)	No	.61
Serine	128.70 (9.97)	143.50 (10.94)	145.90 (9.55)	133.30 (7.10)	No	.56
Glutamine	652.60 (15.25)	714.10 (25.27)	722.20 (33.42)	750.30 (31.82)	No	.12
Ethanolamine	28.82 (1.87)	30.33 (2.41)	33.31 (2.67)	28.32 (3.99)	No	.60
Glycine	335.80 (15.91)	364.80 (17.79)	370.20 (29.40)	339.60 (23.63)	No	.62

Aspartic acid	3.14 (0.37)	2.94 (0.30)	4.69 (1.02)	3.49 (0.51)	No	.19
Sarcosine	0.87 (0.11)	1.22 (0.10)	1.07 (0.20)	1.42 (0.14)	No	.07
Citrulline	61.00 (5.01)	68.61 (3.72)	73.44 (5.94)	63.75 (2.31)	No	.24
Glutamate	81.31 (9.05)	80.79 (5.72)	116.20 (16.73)	82.17 (10.15)	No	.08
β-Alanine	3.08 (0.53)	3.08 (0.32)	4.54 (0.65)	4.10 (0.59)	No	.13
Threonine	110.60 (10.11)	124.80 (7.92)	128.60 (8.44)	105.60 (5.98)	No	.17
Alanine	508.40 (30.31)	584.60 (35.87)	582.10 (27.52)	456.30 (19.09)	Yes	.01
GABA	0.37 (0.05)	0.54 (0.12)	0.89 (0.28)	0.84 (0.17)	No	.17
α-Aminoadipic acid	8.09 (0.44)	7.31 (0.50)	8.40 (0.67)	7.11 (0.59)	No	.32
Proline	76.57 (6.30)	104.80 (12.62)	102.30 (8.31)	80.28 (3.01)	No	.06
Hydroxylysine 2	0.69 (0.09)	0.77 (0.04)	0.68 (0.08)	0.69 (0.07)	No	.75
AABA	3.35 (0.24)	3.22 (0.28)	3.00 (0.21)	4.97 (0.69)	Yes	.005
Ornithine	116.40 (17.57)	177.30 (20.16)	177.60 (15.96)	103.30 (20.32)	Yes	.01
Cystathionine 1	2.25 (0.37)	3.01 (0.63)	1.37 (0.08)	2.97 (0.42)	Yes	.04
Lysine	251.70 (13.36)	263.80 (9.97)	260.80 (10.56)	242.80 (18.79)	No	.67
Cystine	1.12 (0.07)	1.07 (0.07)	1.08 (0.08)	1.03 (0.07)	No	.88
Tyrosine	71.94 (5.89)	90.27 (7.93)	85.09 (6.21)	85.86 (4.08)	No	.24

Methionine	40.44 (2.68)	43.91 (1.82)	46.90 (2.12)	40.73 (2.35)	No	.16
Valine	158.00 (14.05)	179.20 (8.88)	181.40 (11.48)	147.90 (13.46)	No	.15
Isoleucine	73.64 (8.95)	81.83 (4.72)	82.26 (5.49)	66.17 (8.14)	No	.31
Allo isoleucine	0.03 (0.02)	0.07 (0.03)	0.15 (0.08)	0.05 (0.02)	No	.34
Leucine	117.10 (11.33)	129.90 (7.69)	131.20 (8.48)	102.80 (13.03)	No	.18
Phenylalanine	60.65 (4.14)	74.19 (4.81)	70.45 (5.16)	68.94 (4.27)	No	.24
Tryptophan	51.52 (2.94)	51.18 (1.57)	54.07 (2.35)	56.72 (2.36)	No	.31
Serotonin	42.58 (2.98)	44.94 (3.70)	34.21 (1.18)	44.31 (2.19)	Yes	.03

Abbreviations: AABA, α -amino-n-butyric acid; GABA, γ -aminobutyric acid; HI-rTMS, high-intensity repetitive transcranial magnetic stimulation; LI-rTMS, low-intensity repetitive transcranial magnetic stimulation; MI-rTMS, medium-intensity repetitive transcranial magnetic stimulation; OB, olfactory bulbectomy; rTMS, repetitive transcranial magnetic stimulation.

^a Bold indicates statistical significance.

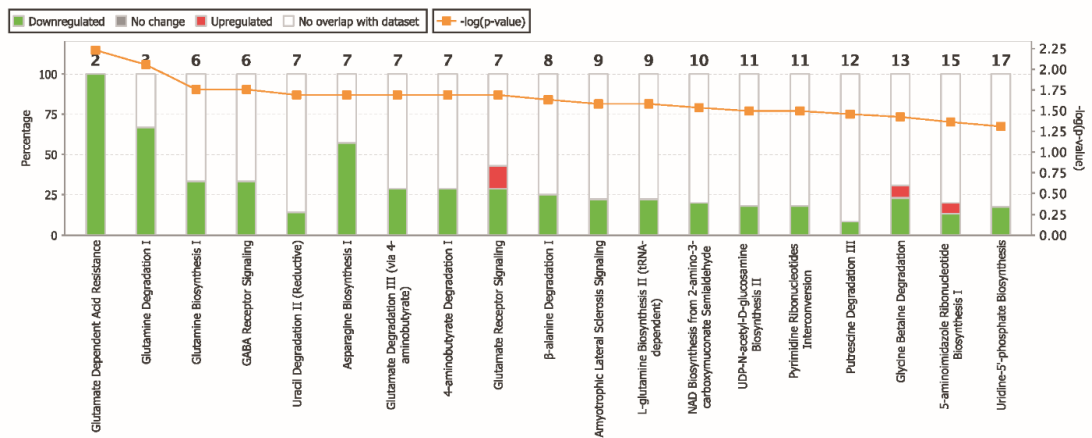
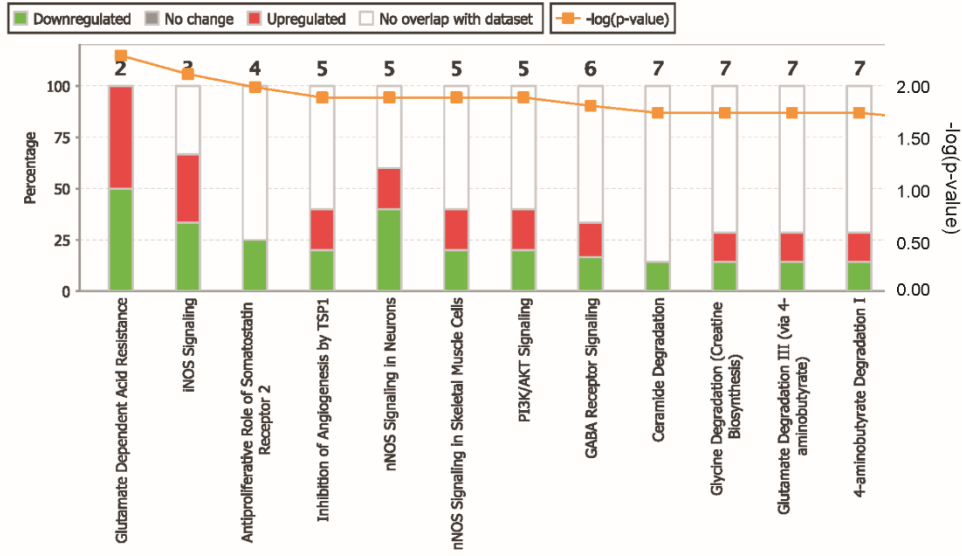


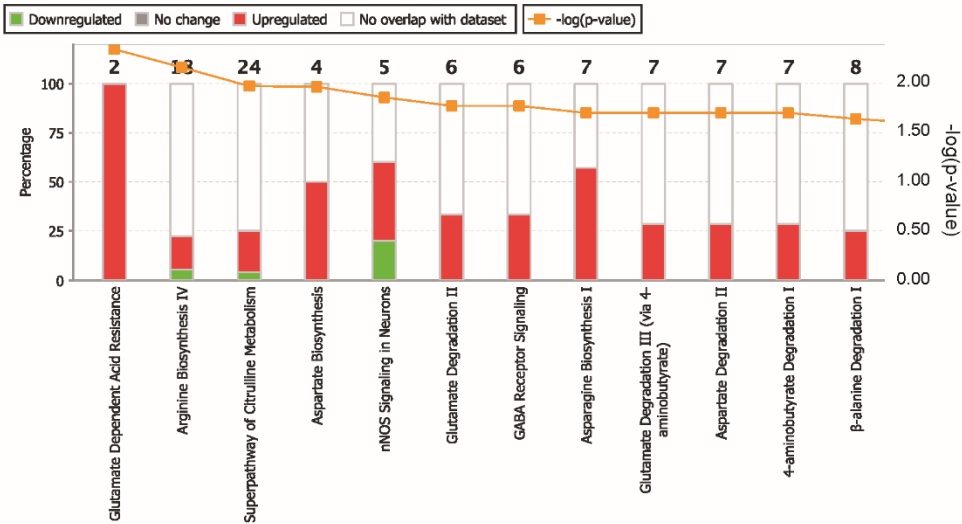
Figure 2.S1 Ingenuity pathway analysis (IPA) revealed that the top four canonical pathways affected by olfactory bulbectomy included glutamate-dependent acid resistance ($p = 5.93 \times 10^{-3}$), glutamine degradation ($p = 8.89 \times 10^{-3}$), glutamine biosynthesis ($p = 1.77 \times 10^{-2}$), and GABA receptor signalling ($p = 1.77 \times 10^{-2}$). Glutamate receptor signalling was also significantly affected by olfactory bulbectomy ($p = 2.06 \times 10^{-2}$). Physiologically, the metabolic changes resulting from olfactory bulbectomy are associated with increased activation of the brain ($p = 2.8 \times 10^{-3}$), reduced inhibition of presympathetic neurons ($p = 2.97 \times 10^{-3}$), and inhibition of synapse maturation ($p = 2.97 \times 10^{-3}$).

Figure 2.S2 (Following Page) IPA analysis of the metabolomics data suggests that rTMS stimulation parameters exert unique physiological effects. For example, LI-rTMS significantly alters metabolites associated with glutamate-dependent acid resistance ($p = 5.93 \times 10^{-3}$), iNOS signalling ($p = 8.89 \times 10^{-3}$), the antiproliferative role of the somatostatin II receptor ($p = 1.18 \times 10^{-2}$), inhibition of angiogenesis by TSP1 ($p = 1.48 \times 10^{-2}$), and nNOS signalling in neurons ($p = 1.48 \times 10^{-2}$) (**Figure 2.S2A**). Mid-intensity rTMS significantly affects some of the same canonical pathways such as glutamate-dependent acid resistance ($p = 1.65 \times 10^{-5}$) and nNOS signalling in neurons ($p = 1.64 \times 10^{-4}$), but also affects arginine biosynthesis ($p = 3.92 \times 10^{-5}$), the citrulline metabolism superpathway ($p = 9.61 \times 10^{-5}$), and aspartate biosynthesis ($p = 9.89 \times 10^{-5}$) (**Figure 2.S2B**). Interestingly, both low-intensity rTMS and mid-intensity rTMS also significantly affect canonical pathways associated with glutamate degradation and GABA receptor signalling (**Figures S2A and B**). Like other rTMS stimulation paradigms, high-intensity rTMS significantly alters glutamate-dependent acid resistance ($p = 5.93 \times 10^{-3}$) and GABA receptor signalling ($p = 1.77 \times 10^{-2}$) (**Figure 2.S2C**). It also affected canonical pathways involved in glutamate degradation ($p = 2.06 \times 10^{-2}$), albeit by altering a different branch of the catabolic pathway. High intensity rTMS also significantly altered canonical pathways associated with histamine biosynthesis ($p = 5.93 \times 10^{-3}$) and ceramide degradation ($p = 2.06 \times 10^{-2}$). Perhaps most importantly, many of the pathways that were downregulated by olfactory bulbectomy, including glutamate-dependent acid resistance, glutamate degradation, and GABA receptor signalling, were upregulated by all forms rTMS. Furthermore, while olfactory bulbectomy appears to largely inhibit many metabolic functions, rTMS appears to exert stimulatory effects of multiple pathways, increasing the concentration of metabolites involved in multiple distinct pathways.

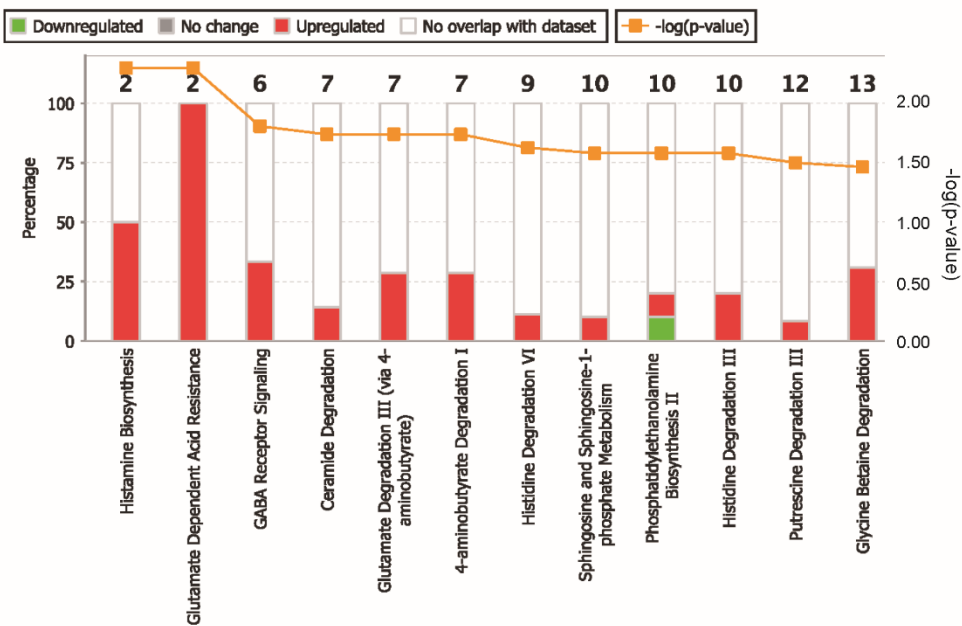
A



B



C



Unpublished Data: Corticosterone and ACTH

Levels of circulating corticosteroids play a large role in the pathology of depression (Krishnan & Nestler 2008), and hypercortisolemia is strongly associated with melancholic and psychotic subtypes of the disorder (Carroll et al. 2007). Following rTMS, in both healthy and depressed patients a decrease in cortisol was observed (Baeken et al. 2009; Evers, Hengst & Pecuch 2001), however this is not always the case as some studies showed no change in cortisol levels (Zwanzger et al. 2003). We therefore decided to investigate the levels of two corticosteroids which are important in the HPA axis, corticosterone and ACTH. The serum extracted as previously described in the Materials and Methods was used in ELISA's to analyse corticosterone (Enzo Life Sciences) and ACTH (Phoenix Pharmaceuticals). These were performed as per the manufacturer's instructions to determine the quantity of these hormones in the blood.

The results were assessed for normality, skewness and kurtosis and the ACTH were not normally distributed (Shapiro Wilk $p < 0.0001$). Though the corticosterone results did pass the tests of normality however did not have equality of error variances (Levene's statistic $F(4,33) = 4.971$ $p = 0.003$). As the assumptions for parametric testing were not met we analysed the data using the non-parametric Kruskal Wallis Test. We found both a significant difference amongst the groups for the ACTH ($H = 10.366$ $Df = 4$, $n = 38$, $p = 0.035$) and corticosterone ($H = 9.778$ $Df = 4$, $n = 38$, $p = 0.044$). A Dunn's multiple comparison test was used to assess differences between the control SHAM/SHAM groups and the different treatments. In the ACTH analysis it was the group that received the OB surgery but a SHAM rTMS treatment that had elevated levels of ACTH in the blood (Figure 2.S3A, $p = 0.0128$). For the corticosterone data only the OB/Hi-rTMS had significantly elevated levels compared to control (Figure 2.S3B, $p = 0.0396$).

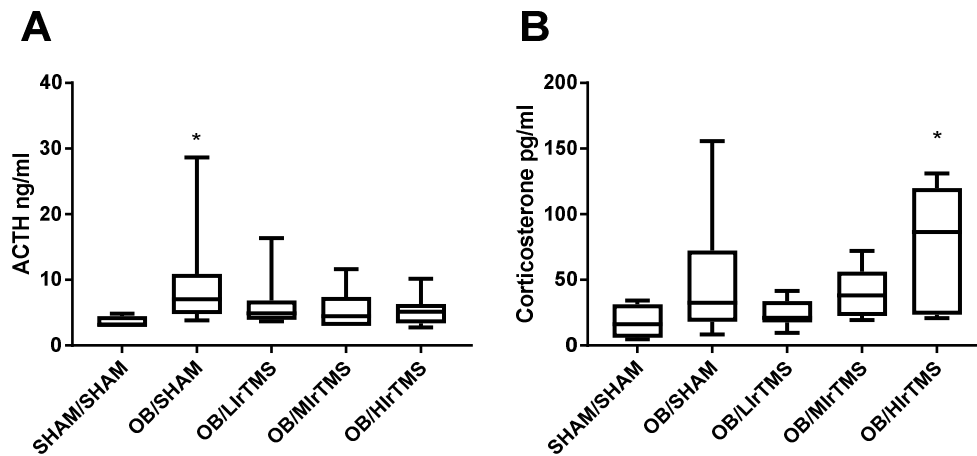


Figure 2.S3 Changes in circulating ACTH and corticosterone after OB and different intensity rTMS. The OB surgery caused elevated levels of ACTH (a) compared to control, whereas HI-rTMS increased levels of corticosterone (b). * $p < 0.05$

The results indicate that both HI-rTMS and OB may be altering the HPA axis. Elevated ACTH after OB confirms previous studies also showing that this model influences the HPA axis (Machado et al. 2012; Marcilhac et al. 1999b), interestingly this did not translate to significantly increased corticosterone levels as well. This may be due to the response times for corticosterone to be elevated after ACTH and therefore we only captured the increase in ACTH and not the subsequent corticosterone elevation (De Souza & Van Loon 1982). As HI-rTMS increased corticosterone this suggests some effect of the stimulation on the HPA axis in these mice, which is in contrast with the known effect of rTMS reducing the levels of cortisol in humans (Evers, Hengst & Pecuch 2001). Research has shown that pentobarbital can initiate a response of the HPA axis (Wu et al. 2015), and though care was taken to extract samples immediately after injection, one aspect we cannot determine from this study is if these results are indicative of resting or stress-induced hormone levels. Nevertheless, the effect of both OB and HI-rTMS on the HPA axis hormones is apparent.

Chapter 3:

Frequency dependent effects of low intensity rTMS in a murine model of Alzheimer's disease

Recently, there has been more interest in using non-invasive brain stimulation techniques for enhancing cognition in Alzheimer's disease to potentially treat this disorder. However, most research into the use of rTMS has been quite limited by a lack of exploration of alternative methods of the stimulation. Therefore, we decided to investigate the effect of LI-rTMS stimulation and different frequency protocols in a preclinical model of AD.

In comparison to Chapter 2 we chose to focus on only a low intensity stimulation. As AD is predominantly a disease of synaptic abnormalities, we chose this intensity due to the success of previous research from our laboratory showing this form of stimulation improves abnormal circuitry while having little effect on normal circuitry. In addition, at subthreshold stimulation it cannot be assumed that the effect of frequency is the same as at suprathreshold stimulation, therefore we decided to investigate both excitatory and inhibitory frequencies of LI-rTMS in this chapter. The parameters used in this study were maintained from previous studies with a 10 minute stimulation for two weeks used to induce chronic changes.

Six and 12 month PS1M146V female mice, a genetic knock in model of AD, and C57BL6/J age-matched controls were stimulated for two weeks with LI-rTMS focused over the hippocampus with one of four different frequencies; two classically excitatory: BHFS and 10Hz and two classically inhibitory: 1Hz and cTBS, or a sham stimulation.

We first wanted to assess whether LI-rTMS could improve the cognition of the AD mice. These effects were assessed in the Open Field Test, Elevated Plus Maze and Morris Water Maze. We found positive effects of the two excitatory frequencies (BHFS and 10Hz) in restoring Morris Water Maze spatial memory deficits. Importantly, this was without having an effect on the anxious phenotype seen in the Elevated Plus Maze, suggesting that the behavioural effect was solely due to an improvement in cognition.

To further explore the potential underlying neurobiological changes that contributed to improved behaviour we assessed outcomes related to structural plasticity and learning

including dendritic spine morphology, neurogenesis and BDNF levels in the hippocampus. The excitatory LI-rTMS reversed the dendritic spine abnormalities seen in this model. However, the stimulation did not improve the decreased neurogenesis and only appeared to have a slight effect on BDNF levels. A separate cohort which received an acute stimulation was used to assess gene expression changes and revealed a potential relationship of the synaptic abnormalities in this model to changes in calcium signalling cascades after excitatory LI-rTMS.

In contrast to the effects of the excitatory stimulations, 1Hz appeared to have little effect while cTBS often worsened measures such as spatial learning in the Morris Water Maze and neurogenesis. Additionally, though the LI-rTMS was also effective in the 12 month PS1 aged mice, in the C57 controls it worsened the memory of the mice. These results stress the importance of parameter consideration when using brain stimulation techniques, as there is a potential for both positive or negative effects.

Overall, this chapter demonstrates the positive effects excitatory LI-rTMS has in an animal model of AD; improving the spatial memory through potentially improving the dendritic spine abnormalities in PS1 mice.

Planned for Publication

Frequency Dependent Effects of Low Intensity rTMS in a Murine Model of Alzheimer's Disease

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Introduction

Repetitive transcranial magnetic stimulation (rTMS), a form of non-invasive brain stimulation, has emerged as a promising technique to target cognitive decline in Alzheimer's disease (AD). Research has shown positive effects of rTMS in facilitating cognitive activities acutely (Cotelli et al. 2008) and also improvements after long term treatment (Ahmed et al. 2012; Devi et al. 2014; Lee et al. 2016; Rabey & Dobronevsky 2016; Zhao et al. 2017b). However, studies have been limited by the treatment parameters used (location, timing, frequency) as they are often the same as for treating depression (i.e. dorsolateral prefrontal cortex (DLPFC) stimulation), which means dissecting out the specific effects of rTMS on mood or cognition is difficult (Ahmed et al. 2012; Padala et al. 2018; Rutherford, Gole & Moussavi 2013).

Low intensity stimulation techniques are an appealing alternative for stimulating the brain because of their ease of application and cost effectiveness. Already pulsed electromagnetic field therapy has been investigated in AD, but with very differing outcomes (Arendash et al. 2012; Dragicevic et al. 2011; Sobel et al. 1996). One reason for this may be because of the lack of focality of these therapies, which are applied to the whole head. We have created small stimulation coils, which are able to focally stimulate rodent brains with low intensity rTMS (LI-rTMS). Stimulation with these coils has been effective in restoring abnormal circuits in the visual system associated with improved visual tracking behaviour (Rodger et al. 2012). In addition, dependent on frequency, LI-rTMS can regulate various neurobiological processes such as intracellular calcium flux, Brain Derived Neurotrophic Factor (BDNF) expression and neuronal neurite branching both *in vitro* and *in vivo* (Grehl et al. 2015; Makowiecki et al. 2014). Humans with AD and rodent models of AD have disruptions in intracellular calcium signalling (Ito et al. 1994; Tu et al. 2006), synaptic plasticity (Auffret et al. 2010; Koch et al. 2012; Pozueta et al. 2013) and BDNF levels (Peng et al. 2009; Qin et al. 2017). Therefore LI-rTMS may provide a novel method of targeting this synaptic dysfunction and ameliorating cognitive deficits.

Genetic mutations discovered in familiar AD have been critical in translating AD to rodent models. In this paper we used a human presenilin-1 (PS1) knock in model that expresses the mutation M146V. PS1 is a major component of γ -secretase, which cleaves amyloid precursor protein to the plaque forming amyloid beta, but it also has many

other functions in development and memory (Ho and Shen 2011). Although mice with the mutation do not exhibit the typical amyloid plaque pathology they do accumulate A β in brain tissue, thus PS1M146V mice have been used effectively to demonstrate behavioural and neurobiological phenotypes of AD. Mice develop deficits in spatial memory and fear learning (Sun et al. 2005; Wang et al. 2004), and abnormal hippocampal long term potentiation (LTP), dendritic spine morphology and neurogenesis (Auffret et al. 2010; Sun et al. 2014; Wang et al. 2004; Zhang et al. 2015a). Recent research has linked these changes to a disturbance in the PS1 function in Calcium regulation, which causes an accumulation of endoplasmic reticulum (ER) Calcium (Stutzmann et al. 2006; Sun et al. 2014; Tu et al. 2006; Zhang et al. 2015a). Unlike other AD models which develop extreme pathology at young ages (Liu et al. 2015), the phenotype in this model develops progressively with age like human AD (Gold & Budson 2008; Karantzoulis & Galvin 2011), making it an appropriate model to examine the effectiveness of treatments across the course of the disorder.

To examine whether LI-rTMS could treat the cognitive decline in AD we looked at different LI-rTMS frequencies and their effect in the PS1M146V model of AD. We investigated both behavioural and neurobiological changes as treatment outcomes. After 2 weeks of stimulation we found positive effects of the frequencies which are excitatory in high-intensity stimulation (Biomimetic High Frequency (BHFS) and 10Hz) (Hallett 2007; Rodger et al. 2012) in restoring memory deficits and dendritic spine abnormalities, whereas frequencies described as inhibitory (Hallett 2007; Huang et al. 2011) were less effective: 1Hz induced partial improvement and continuous Theta Burst Stimulation (cTBS) had either no effect or, in some cases, worsened the parameter. In contrast, LI-rTMS did not improve the anxious phenotype of the mice or the loss of neurogenesis.

Materials and Methods

Animals

Six month and 12 month female PS1M146V knock-in mice (PS1) were used in this study with C57Bl/6J (C57) mice as aged matched controls. Mice were kept in cages of 4-5 on a 12:12h light dark cycle with free access to food and water. All procedures were authorized by the Comité National d'Ethique pour les Sciences de la Vie et de la Santé

N°5 (N° 1493-02), in accordance with the European Communities Council Directive 2010/63/EU and regulations of the NIH.

Study Design and Groups

The effect of both genotype and LI-rTMS treatment was examined using both behavioural and biological measures. Six month PS1 (n=60) and C57 (n=60) mice were randomly divided (n=12 per group) into receiving SHAM (PS1-SHAM, C57-SHAM) or one of four different stimulation frequencies, BHFS (PS1-BHFS, C57-BHFS), 10Hz (PS1-10Hz, C57-10Hz), 1Hz (PS1-1Hz, C57-1Hz) and cTBS (PS1-cTBS, C57-cTBS). For detailed descriptions of these frequencies see the LI-rTMS Treatment section below. The 12 month mice (n=12 per group) only received either SHAM (12 Month PS1-SHAM, C57-SHAM) or BHFS (12 Month PS1-BHFS, C57-BHFS). All groups underwent one week of habituation and two weeks of stimulation. They were then tested in the behavioural measures (Open Field Test, Elevated Plus Maze and Morris Water Maze) over another three weeks. Following the end of this six week experimental period these treated mice were randomly divided into subsets (n=4 per group) for neurobiological analysis of hippocampal neurogenesis, dendritic spines and BDNF levels. An additional group of 6 month PS1 and C57 mice were used for analysis of gene expression changes, treated with either SHAM or BHFS stimulation for 3 days (n=4 per group).

LI-rTMS Treatment

Mice were handled for one week prior to stimulation to familiarize them to the procedures used. They then underwent two weeks of stimulation, which occurred between 10am-4pm using a low intensity rTMS coil held over the dorsal hippocampus area (Figure 3.1A-C). The characterization of this coil has been previously described (Makowiecki et al. 2014; Rodger et al. 2012). Briefly, a custom built copper circular coil (8mm outer diameter, 6mm inner) was powered by an electromagnetic pulse generator (EC10701, Global Energy Medicine, Perth, Australia). These coils deliver a 12mT pulse, with the estimated intensity at the dorsal hippocampus of <4mT (Heath et al. 2018; Makowiecki et al. 2014). Two different excitatory frequencies, BHFS and 10Hz, and two different inhibitory frequencies, 1Hz and cTBS, (Figure 3.1D) were randomly to both C57 and PS1 mice. For each frequency, stimulation was given continuously over a 10 minute period. This resulted in a total of 6000 pulses for 10Hz stimulation and 600 pulses for 1Hz stimulation. Continuous theta burst stimulation comprises trains of 3 pulses at 50Hz

repeated at 5Hz, for a total of 9000 pulses. Biomimetic high frequency stimulation was developed to replicate endogenous patterns of electrical fields around activated nerves and consists of 59.9ms trains of 20 pulses, repeated at 6.67Hz for the first minute, 10.1Hz for 8 minutes and 8.75Hz for the final minute, for a total of 112,000 pulses (Rodger et al. 2012).

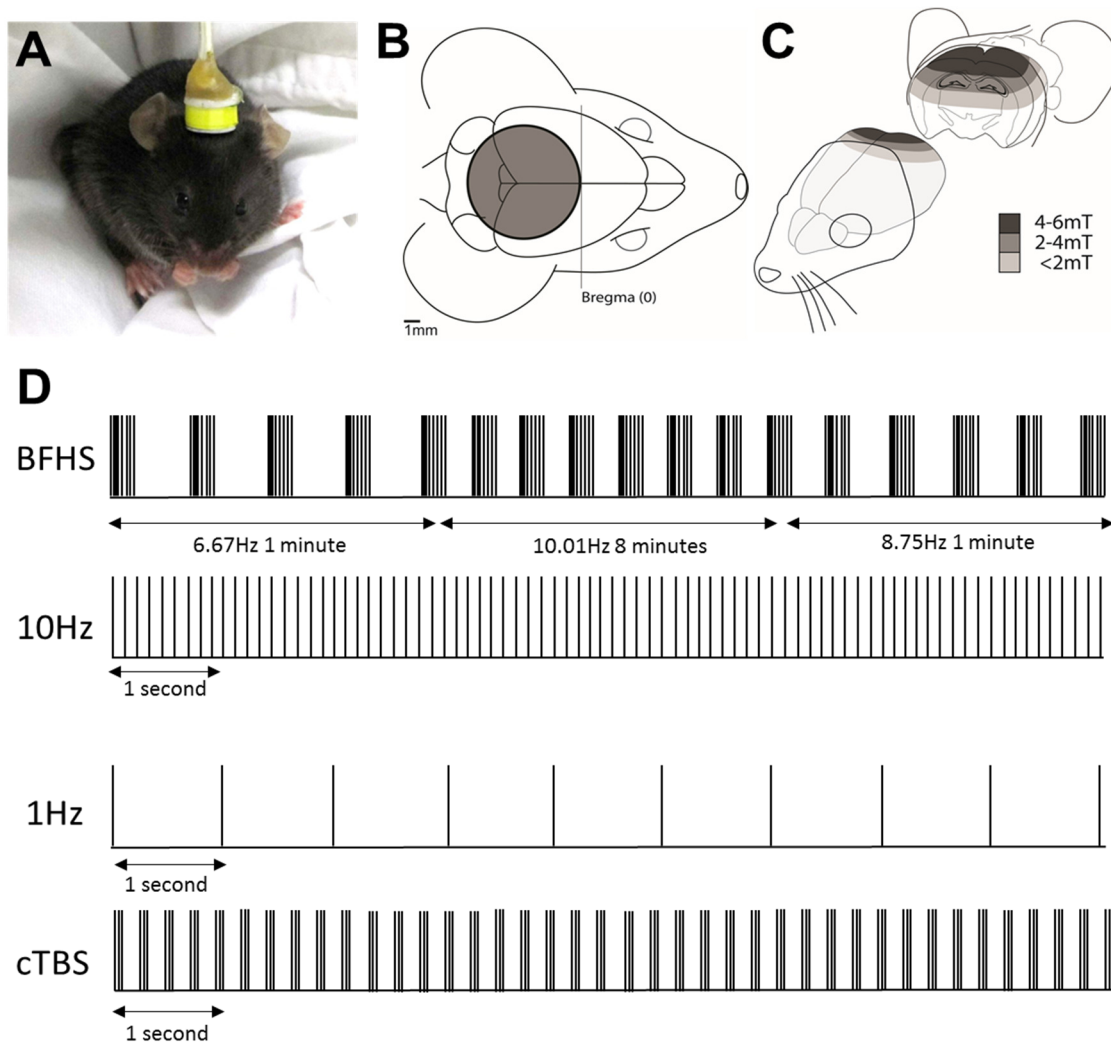


Figure 3.1 LI-rTMS technique in rodent model. Small rodent sized coils (A) were used to target an area directly above the location of the hippocampus (B). The magnetic field depth of these coils drops off from the base of the coil but still would be reaching levels of 2-4mT at the dorsal hippocampus (C). Mice received two weeks of LI-rTMS treatment with one of four different frequency stimulations (D) or a SHAM treatment. BHFS and 10Hz are consider excitatory and 1Hz and cTBS are considered inhibitory.

Behaviour Testing

After 2 weeks of stimulation, the mice were subjected to behavioural tests not only to assess learning and memory but also anxiety related phenotypes. Each test was performed on a separate day during the light phase of the cycle and recorded on Ethovision (EthoVision XT 10) software, unless otherwise stated.

Open Field Test

The open field test is used to measure activity in a novel field. The square arena was 50cmx50cm with dark walls and a white base. Mice were placed into the top right hand corner of the arena and allowed to explore for 10 minutes. Between each mouse the arena was cleaned to eliminate odours. Distanced travelled and time spent in designated inside and outside zones over the 10 minutes was calculated to assess motor activity of the animals.

Elevated Plus Maze

The following day mice were tested in the Elevated Plus Maze, used to examine anxiety like phenotypes. The maze was raised 100cm from the floor with two open and two closed arms measuring 40cm in length each, which were divided into zones 10cm long. The mice were placed into the centre of the arena facing an open arm and allowed to explore for 10 minutes. Between each mouse the maze was cleaned to eliminate odours. The percentage time spent in the open arms (Total Time in Open Arms/ (Total Time in Open +Closed Arms)) was calculated, as well as the percentage of times the mouse entered each arm and travelled to the furthest zone.

Morris Water Maze

The Morris Water Maze (MWM), first developed by Morris (1984), is a classical behavioural test used to examine spatial learning and memory. The arena used measured 120cm in diameter and was filled with water kept at 22°C (+/-1). Three large shapes were hung around the room for navigation with no other visual cues visible. For five days, four trials were performed each day, 10 minutes apart. For each trial the starting location was changed, but the platform location always remained the same. The mice were given 90 seconds to find a platform hidden 1cm under the surface. If after the 90 seconds they had not found the platform they were guided there and stayed on the platform for 20 seconds. On the fifth day 10 minutes after the final trial the mice underwent a probe test. For the probe trial, the platform was removed and the mice searched for 60 seconds.

For the learning phase the distance travelled during the trial was calculated, and for the probe test the time spent in each quadrant and the velocity of each mouse was recorded. Also, using methods adapted from Illouz et al. (2016), an experimenter

blinded to the genotype or treatment of the animals classified the swimming patterns of the mice into nine different categories. These categories were assigned a value from 1-6 according to their hypothesized use of higher cognitive processes: Direct=6, Corrected=5, Focused=4, Chaining, Accidental Circling or Circling=3, Random=2 and Thigmotaxis or Passivity=1 (For examples see Figure 3.4C). The values were used to calculate an average cognitive score for individual mice on each day of learning.

The following week the mice were tested in the cued platform MWM to assess differences in ability to orientate and swim to a specific place (the platform) between the different genotypes and ages. Trials for conducted four times a day for five days, this time 20 minutes apart. All cues were removed from the room and the platform marked with an object. For each trail the location of the platform was changed. For this test the distance to the platform for each trial was recorded.

Neurobiological Analysis

Mice from each group were randomly separated into three subgroups (n=4 each group) for analysis of dendritic spines, neurogenesis and hippocampal Brain Derived Neurotrophic Factor (BDNF). The subset of mice for the analysis of neurogenesis were injected with 5-ethynyl-2'-deoxyuridine (EdU Invitrogen 50mg/kg) once a day for three days at the beginning of their second week of stimulation. At the end of behavioural experimentation all mice were euthanized (Pentobarbital 300mg/Kg i.p.) and either perfused with 50ml of 4% paraformaldehyde in 0.1 Phosphate Buffer (Golgi staining and neurogenesis immunohistochemistry) or the hippocampus rapidly dissected on ice and stored at -80°C (BDNF Enzyme-Linked Immunosorbent Assay (ELISA)).

Golgi Staining

The first subset of mice was used for Golgi analysis using the NovaUltra kit (IHC World) in accordance with the manufacturer's instructions. Perfused brains were immersed in the Golgi impregnation solution for 10 days. The brains were then washed in PBS for two days, sectioned at 100µm using a vibratome and incubated with the post impregnation solution for 10 minutes. The slices were then dehydrated with 70, 90 and 100% ethanol, cleared in xylene (2x10 minutes) and mounted onto slides with Mowiol (Sigma, France). Neurons in the CA1 were analysed from both the left and right hippocampi in 3 slices per brain, sampled from the anterior, middle and posterior dorsal hippocampus, for a

total of 6 neurons per animal. Z-stack images were taken at x63 of both apical and basal dendrites (Leica DM6000) and spines counted on a 10-30µm length of dendrite, which was clearly visible and did not cross other labelled processes. Based on visual characteristics, the spines were categorised into subgroups, stubby, thin and mushroom.

Neurogenesis Immunohistochemistry

The effect of LI-rTMS on neurogenesis in the dentate gyrus was observed using EdU labelling. The fixed brains were sectioned using a vibratome at 35µm. EdU staining was visualised using the Click-iT reaction kit (Invitrogen Click-iT[®] EDU Alexa Fluor[®] 555), this was performed in combination with immunohistochemistry for NeuN and Hoechst to identify the newly born cells that had differentiated into neurons. Sections were washed with 0.1% Triton in PBS (0.1% PBST) and then permeabilised and blocked for non-specific staining with 20% normal donkey serum in 0.1% PBST for one hour at room temperature, prior to incubation overnight at 4°C with the primary antibody mouse anti-NeuN (1:300) in 20% normal donkey serum in 0.1% PBST. The following day sections were washed, incubated at room temperature with 488-conjugated donkey anti-mouse (1:400) and the nuclear dye Hoechst (1:1000) in 0.1% PBST for three hours, and re-washed for EdU visualisation with the Click-iT kit according to the manufacturer's instructions. Briefly, sections were further permeabilised in 0.5% Triton in PBS for 30 minutes and washed twice in 3% BSA in PBS. The reaction cocktail was prepared as instructed, using the reaction buffer, copper sulphate, AF555 and reaction additive. Sections were incubated in the reaction cocktail for 30 minutes, washed twice in 3% BSA in PBS and then once in PBS. Sections were mounted onto Supafrost slides (Thermo Scientific) and cover slipped with Mowiol (Sigma, France).

EdU labelled cells were counted in the granule layer of the dentate gyrus of both the left and right hippocampi. Co-staining of EdU labelled cells with NeuN was noted however, the total number of NeuN cells were not quantified. Sections were counted from a one in four series, with the length of the granule layer measured to normalise the values to the size of the hippocampus.

BDNF ELISA

For the ELISA, one hippocampus per animal was homogenised in 250µl acid extraction buffer, consisting of 1M NaCl, 0.1% Triton and 50mM Sodium Acetate (pH 4.0), with a protease inhibitor cocktail added before use, sonicated and centrifuged for 30 min at 10000g at 4°C. The supernatants taken and stored at -80°C. Protein concentration was measured using the DC protein analysis kit (Bio-Rad). Protein extracts were neutralised in 1:4 neutralisation buffer (0.1M Phosphate Buffer) and diluted to 1:100 for the BDNF ELISA. Levels of BDNF were analysed using the Chemokine BDNF ELISA assay, samples were measured in duplicate in accordance with the manufacturer's instructions.

qRT-PCR

Changes in gene expression, triggered by excitatory LI-rTMS, were examined in a different cohort of PS1 and C57 mice which received either SHAM or BHFS stimulation for three days (n=4 each group). A shorter time frame was chosen compared to previous experiments to investigate the stimulation-induced changes in the expression of genes, which may contribute to the long-term effects seen (e.g. spine morphology, neurogenesis). Five hours following the final stimulation the mice were euthanised and the hippocampi rapidly dissected on ice. Total RNA was extracted from each hippocampus using Trizol (Life Technologies) according to manufacturer's instructions and stored at -80°C.

cDNA was transcribed from 400 ng total RNA using the RT² Easy First Strand cDNA Synthesis Kit (Qiagen). For each sample, the resulting cDNA was applied to the RT² Profiler™ PCR Array Mouse Neurogenesis (Qiagen) and amplified with a LightCycler480 System. Results were analysed using the Qiagen RT² Profiler PCR array data analysis centre (v3.5) using the housekeeping gene, Actb. Normalized mean expression levels ($\log_2(2^{-\Delta Ct})$) were used to determine differentially expressed genes between each group. An Overrepresentation Enrichment Analysis on significantly regulated genes was performed using Webgestalt 2013 (Zhang et al. 2005), mapping to both Gene and Phenotype Ontology.

Statistical Analysis

All data was able to meet the assumptions of normality as assessed by examining the skewness, kurtosis and the Shapiro Wilk value of the calculated residuals and there were

no violations of the homogeneity of variance as assessed using Levene's statistic. Data from the 6 month mice were tested for significance using either a Two Way ANOVA examining the effect and interaction of genotype or treatment (LI-rTMS), or a Repeated Measures Two Way ANOVA when a within subject factor was involved (Time or Quadrant in the MWM or Dendritic Spine Type). For the Repeated Measures ANOVA when the assumption of sphericity could not be met the Greenhouse Geisser significance test was used instead. Data from 12 month old mice were analysed by a Three Way ANOVA or Repeated Measures Three Way ANOVA (genotype, treatment, age) because of the addition of the age variable. For all post-hoc pairwise comparisons a Tukey adjustment was used with significance at $p < 0.05$. All statistics were performed using SPSS (version 22, IBM, France) and graphs produced using GraphPad Prism (version 7). For the qRT-PCR results, changes in gene expression levels were compared using a two sample t-test which was performed using the Qiagen Data Analysis Centre.

Results

Open Field Test

As future tests rely on the ability to move and explore the Open Field Test was used to assess the motor activity of the animals. There was no difference in activity between genotypes and after the two weeks of stimulation (Figure 3.2A). This lack of difference was also observed at 12 months of age (Figure 3.2B). Analysis of time spent in the inside and outside zones did not show any statistically significant differences (data not shown).

Elevated Plus Maze

The elevated plus maze is used to assess anxious phenotypes in animals. When examining the percent time spent in the open arms, PS1 mice were more anxious at both 6 and 12 months (6 Month Genotype Effect $F(1,109)=12.026$ $p=0.001$, 12 Month Genotype Effect ($F(1,43)=6.144$ $p=0.017$). But post hoc analysis revealed no significant differences (Figure 3.3A,B). Therefore, analysis was undertaken to discriminate between

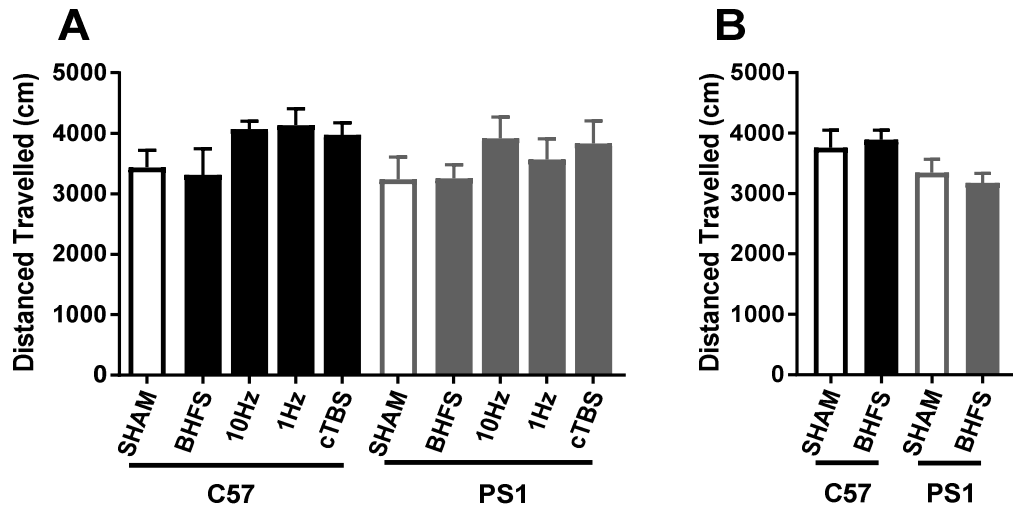


Figure 3.2 Activity in the Open Field Test. Total distance travelled (cm) over a ten minute period was assessed in both 6 month (A) and 12 month (B) mice. There was no difference amongst the groups.

different zones of the open and closed arms, as zones in the open arms closer to the centre can be considered “less stressful” than zones further toward the end. The percentage of times the mouse entered the open arms and went to these more anxiogenic end zones was calculated (Figure 3.3E,F). Again the PS1 mice showed more anxiety than the C57 mice (Effect of Genotype $F(1,109)=64.731$ $p<0.001$), but now there was an effect of the LI-rTMS treatment depending on the genotype (Treatment x Genotype Interaction $F(4,109)=2.578$ $p=0.041$). From post hoc analysis the C57-SHAM mice went more times to the end zones than the PS1-SHAM, PS1-10Hz PS1-1Hz and PS1-cTBS mice ($p<0.05$ each). Additionally, the percentage of open-arm entries where the PS1 mice treated with BHFS or 10Hz went to the end zones was not significantly different from the C57-SHAM mice; but neither were they significantly different from the PS1-SHAM mice, suggesting a small but not significant effect of the treatment in these animals. The anxious phenotype of the PS1 mice extended to the 12 month group of animals (Effect of Genotype $F(1,43)=9.483$ $p=0.004$), with post hoc analysis revealing both PS1-SHAM and PS1-BHFS mice travelled to the end zones less than C57-SHAM ($p<0.05$). To eliminate the possibility that these changes occurred because of overall lower exploration by these animals the same measure were examined in the closed arms (Figure 3.3 C,D). For both the 6 and 12 month group neither the genotype of the animals

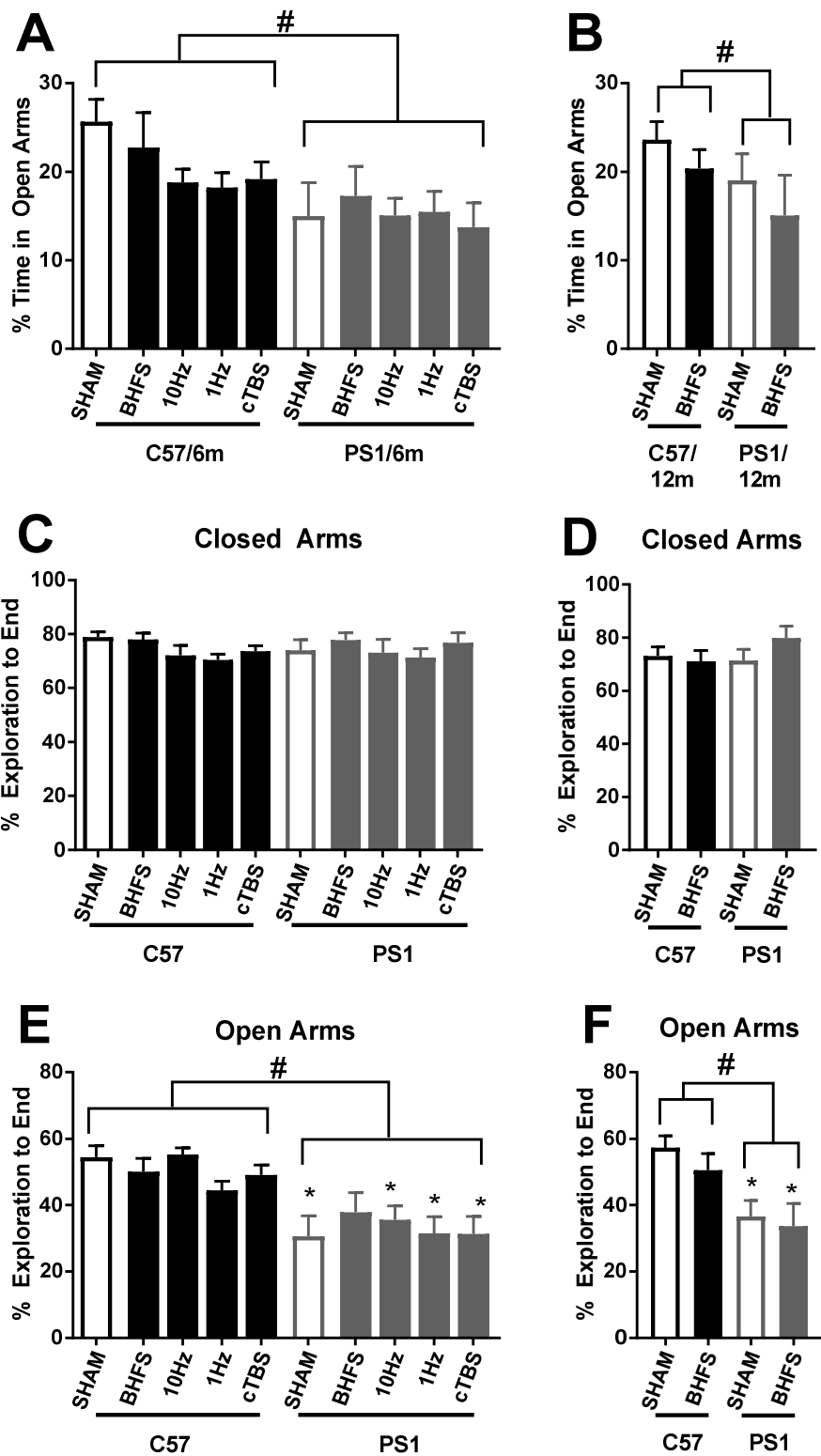


Figure 3.3 PS1M146V mice have an anxious phenotype that is not affected by LI-rTMS. The Elevated Plus Maze was used to assess anxiety behaviours by calculating the percentage of total time spent in the open arms. PS1M146V mice spent less time in the open arms at both 6 (A) and 12 months (B). This effect was further investigated by dividing the arms into zones and calculating the times the rodent entered each arm and travelled to the furthest zone (% Exploration to End). There was no difference in closed arm exploration (C,D) whereas in the open arms the PS1 mice went to the end “more anxious” zones less at both 6 (E) and 12 months (F). None of the LI-rTMS frequencies had any effect on both measures of anxiety (A,B,E,F) (# $p < 0.05$ Genotype Effect, * $p < 0.05$ vs C57-SHAM).

or the stimulation given had an effect on the exploration of the closed arms. Suggesting that the differential exploration of end zones only occurs in the open arms which have these “more stressful” zones, validating this analysis technique.

Morris Water Maze

Learning Phase

Spatial learning and memory were assessed using the Morris Water Maze. All the animals across the five days of learning reduced their distance taken to reach the platform (6 Month Effect of Day, Greenhouse Geisser $F(3.502,384)=175.787$ $p<0.001$, 12 Month Effect of Day $F(4,152)=56.542$ $p<0.001$) and for each individual group there was a reduction the distance covered comparing Day 1 to Day 5 ($p<0.001$; Figure 3.4A,B), suggesting that mice in all groups had learned the platform’s location.

To further examine the learning of these animals, swimming patterns of the mice were classified using a similar classification system to Illouz et al. (2016). Search strategies were given a particular score, depending on their use of higher cognitive functions, resulting in a cognitive score for each day (for more detail see methods and Figure 3.4C). All the animals progressed from a lower cognitive score to a higher cognitive score over the five days (6 Month Effect of Time, Greenhouse Geisser $F(3.682,384)=126.346$ $p<0.001$; 12 Month Effect of Time $F(4,152)=59.813$ $p<0.001$), showing significant improvement for all groups between days 1 to 5 ($p<0.001$; Figure 3.4D,E). On the first day the PS1 mice overall had slightly lower cognitive score than the C57 mice (Effect of Genotype $F(1,97)=3.915$ $p=0.051$) and the type of stimulation given effected the cognitive score on days 1, 2 and 3 (Effect of Treatment Day 1: $F(4,97)=2.906$ $p=0.026$, Day 2: $F(4,97)=7.21$ $p<0.001$, Day 3: $F(4,97)=7.919$ $p<0.001$). Specifically, independent of genotype, mice had a lower cognitive score on days 2 and 3 after cTBS stimulation (C57-SHAM v C57cTTBS $p<0.05$, PS1-SHAM v PS1-cTBS $p<0.05$). But the effect did not continue for the final two days. Also, at 12 months of age the mice had a lower cognitive score than the 6 month mice on the first day, independent of their genotype or treatment (Effect of Age Day 1 $F(1,80)=5.248$ $p=0.025$). However, this effect was only seen on the first day, further training over the subsequent days improved their learning to be level with the six month mice.

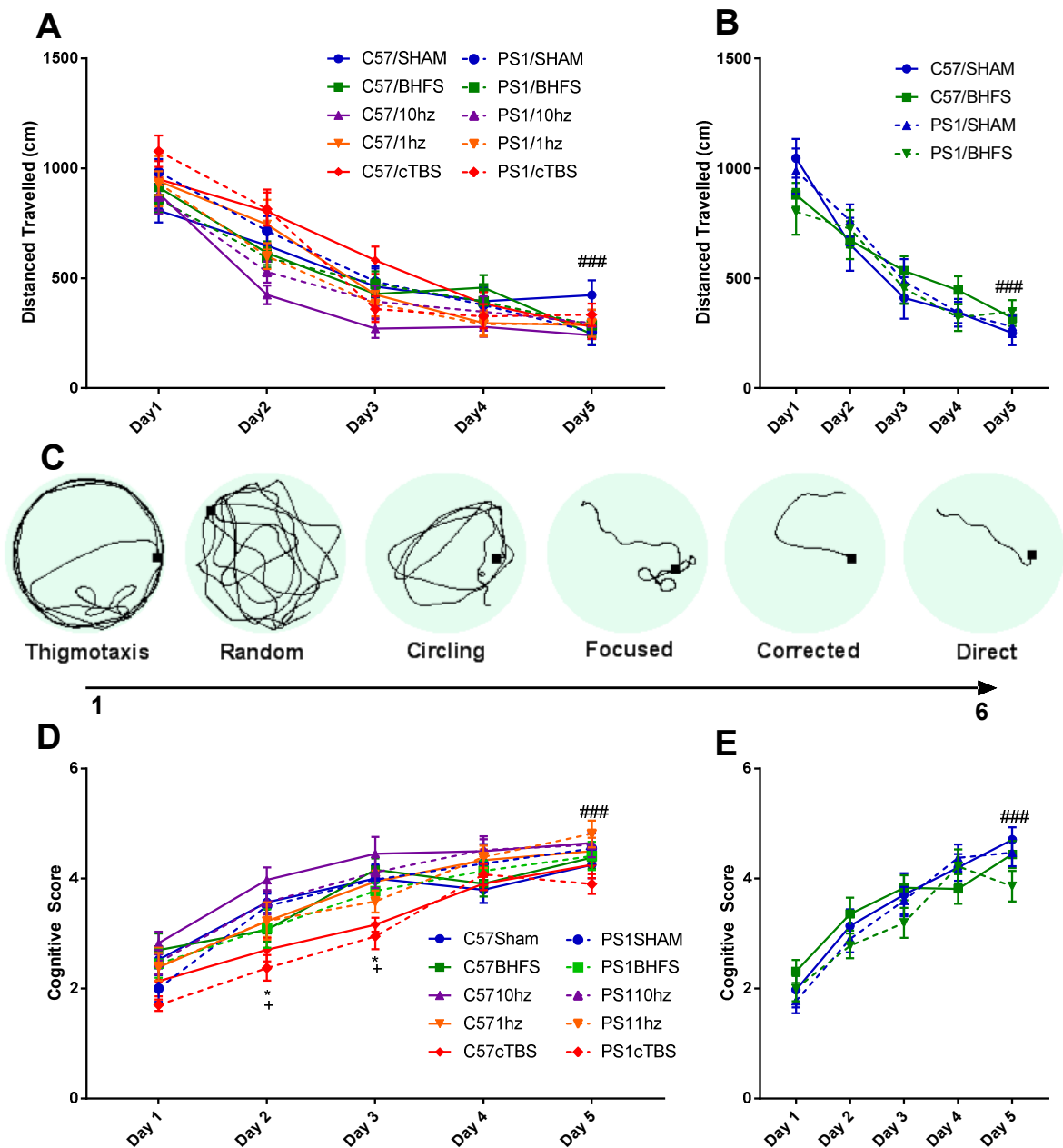


Figure 3.4 Normal spatial learning was affected by cTBS stimulation. The Morris Water Maze was used to assess spatial learning as mice must learn to navigate to a hidden platform using spatial cues. Over the five learning days both PS1 and C57 mice learnt to swim to the platform at 6 (A) and 12 months (B) as measured by their distance taken to reach the platform (cm). To further analyse spatial learning swimming patterns over the five days were categorised into groups and depending on the use of higher cognitive processes assigned numbers on a scale from 1 to 6 (C). These classifications were then used to calculate a cognitive score for each day of learning. From day one to day five all groups improved their cognitive score, however on days two and three the C57 and PS1 mice having received cTBS stimulation were negatively affected (d). On day one the 12 month group also had lower cognitive scores but these improved over the five days (E). ### p<0.001 vs Day 1, * p<0.05 vs C57-SHAM, + p<0.05 vs PS1-SHAM.

Probe Test

Ten minutes after the final learning trial the mice were subjected to a probe test to determine their memory of the platform location. The platform was removed, and the mice searched for the platform for 60 seconds. The time spent in each quadrant can be

used to assess the memory of the platform location, as mice which remember the location have a preference for the target quadrant over the other three quadrants. This preference was revealed in this study, (Effect of Quadrant Greenhouse Geisser $F(2.593,288)=73.046$ $p<0.0001$), and was influenced by both the mouse genotype and the stimulation received (Quadrant x Treatment, Greenhouse Geisser $F(10.371,288)=3.177$ $p=0.001$ Quadrant x Genotype, Greenhouse Geisser $F(2.593,288)=5.641$ $p=0.002$; Figure 3.5B). In sham-treated groups, the C57 mice spent significantly more time in the north (target) quadrant than any of the other three (North v South, North v East & North v West $p<0.01$), consistent with knowing the location of the platform. In contrast, the PS1-SHAM mice spent about 15s in each quadrant, which is at the level of chance (4 quadrants over 60s), indicating that they did not remember where they should be searching for the platform.

Focusing solely on the time spent in the target (north) quadrant as an indication of memory, at 6 months, overall C57 mice spent greater than chance (15s) in the target quadrant, compared to PS1 mice who overall demonstrated significantly impaired memory (Effect of Genotype $F(1,96)=16.414$ $p<0.001$). The LI-rTMS stimulation had a significant effect on this measure (Effect of Treatment $F(4,96)=5.345$ $p=0.001$), however this was dependent on the genotype of the mice as it appears only the PS1 mice which had a behavioural deficit responded in a frequency-dependent manner to the stimulation (Genotype x Treatment interaction $F(4,96)=2.502$ $p=0.047$ Figure 3.5C). Specifically, PS1-SHAM mice spent significantly less time in the target quadrant than the C57-SHAM controls ($p<0.05$), whereas both BHFS and 10Hz treatments increased the time these animals spent in the target quadrant (PS1-SHAM v PS1-BHFS $p<0.01$, PS1-SHAM v PS1-10Hz $p<0.01$), but cTBS stimulation did not (C57-SHAM v PS1-cTBS $p<0.05$). Interestingly, though 1Hz treated group did not show a significant improvement from PS1-SHAMs, they were no longer significantly different from C57-SHAM, indicating that these mice also had a preference for the target quadrant ($F(3,33)=7.108$ $p=0.005$, North v South and East Quadrant $p<0.001$, data not shown). Overall, LI-rTMS improved spatial memory in PS1 mice depending on the frequency.

For mice aged 12 months, the time spent in the target quadrant was dependent on both the genotype and treatment (Genotype x Treatment interaction $F(1,38)=18.979$ $p<0.001$, Figure 3.5D). The PS1-SHAM mice still showed worse memory than the

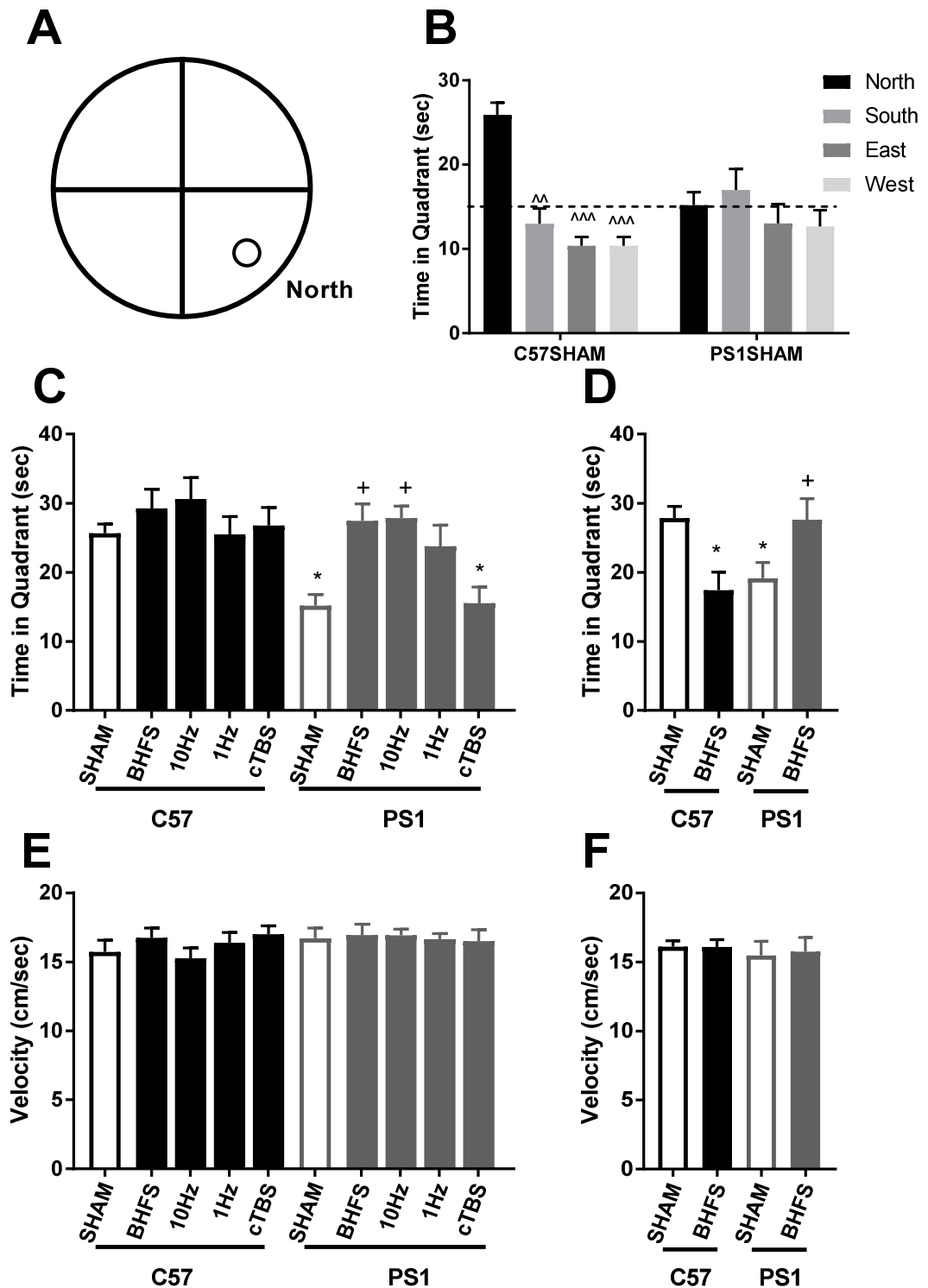


Figure 3.5 LI-rTMS improves spatial memory in PS1 mice. Ten minutes following the final learning trial spatial memory was assessed using the probe test in the Morris Water Maze. As the platform was removed time spent in each quadrant was recorded to assess the memory of the platform location, the northern quadrant was designated as the quadrant where the platform was previously (A). C57-SHAM mice showed a preference for the target North quadrant, whereas PS1-SHAM mice do not have this preference (B) and did not differ from chance (15 seconds, dashed line). Time in the target quadrant was improved after BHFS and 10Hz stimulation at 6 months of age (C) whereas at 12 months (D) BHFS did improve the memory of the PS1 mice it worsened the memory of the C57 mice. To confirm these differences were not due to an inability to perform the test velocity was assessed at both age groups and showed no differences (E,F). $^{^^} < 0.01$, $^{^^^} p < 0.001$ vs North, * $p < 0.05$ vs C57-SHAM, + $p < 0.05$ vs PS1-SHAM.

C57-SHAMs ($p < 0.05$), and similar to the younger mice BHFS treatment appeared to improve memory in these PS1 mice so that they spent more time in the target quadrant ($p < 0.05$) and were equal to C57 mice. However surprisingly, BHFS stimulation worsened the spatial memory of C57 animals ($p < 0.05$).

Importantly, none of these results were influenced by the mouse's ability to perform the task as neither genotype, treatment or age had any effect on the animals swimming speed (Figure 3.5E,F). This was further clarified using the Cued MWM where none of the variables in this study influenced the ability of the mouse to perform the task (Supplementary Data Figure 3.S1)

Dendritic Spines

Dendritic spine density and type classification were quantified on both the apical and basal dendrites of CA1 pyramidal neurons in the hippocampus. In the apical dendrites, overall spine density did not change between the 6 month and 12 month age groups, nor following LI-rTMS treatment (Figure 3.6A,B). However, there were differences in the ratio of spine type (Effect of Spine Type $F(2,56)=7.659$ $p=0.001$). In non-stimulated mice, C57-SHAM group had a significantly greater percentage of mushroom spines than thin and stubby (Effect of Spine Type $F(2,6)=16.551$ $p=0.004$, Mushroom v Thin $p < 0.01$, Mushroom v Stubby $p < 0.001$), whereas the PS1-SHAM group had a lower percentage of mushroom than thin or stubby spines (Effect of Spine Type $F(2,6)=12.063$ $p=0.008$, Mushroom v Thin $p < 0.0001$, Mushroom v Stubby $p < 0.005$, Figure 3.6C). Comparatively, in the basal dendrites there was no difference between the percentage of mushroom spines (Supplementary Data Figure 3.S2). As mushroom spines are thought to be involved in memory formation and retention we decided to focus on their density in the apical dendrites for comparisons between the different treatments.

The percentage of mushroom spines was significantly decreased in the 6 month PS1 animals compared to C57 (Effect of Genotype $F(1,28)=8.804$ $p=0.006$ Figure 3.6E). Considering the percentage of spines that were mushroom-like, the different stimulation frequencies altered this percentage (Effect of Treatment $F(4,28)=3.321$ $p=0.024$) but in a way that was dependent on the genotype (Genotype x Treatment $F(4,28)=8.812$ $p < 0.001$). Post hoc analysis revealed that there was a lower percentage of mushroom spines in the PS1-SHAM mice compared to the C57-SHAM mice ($p < 0.001$). After BHFS, 10Hz and 1Hz stimulation the percentage of mushroom spines was increased

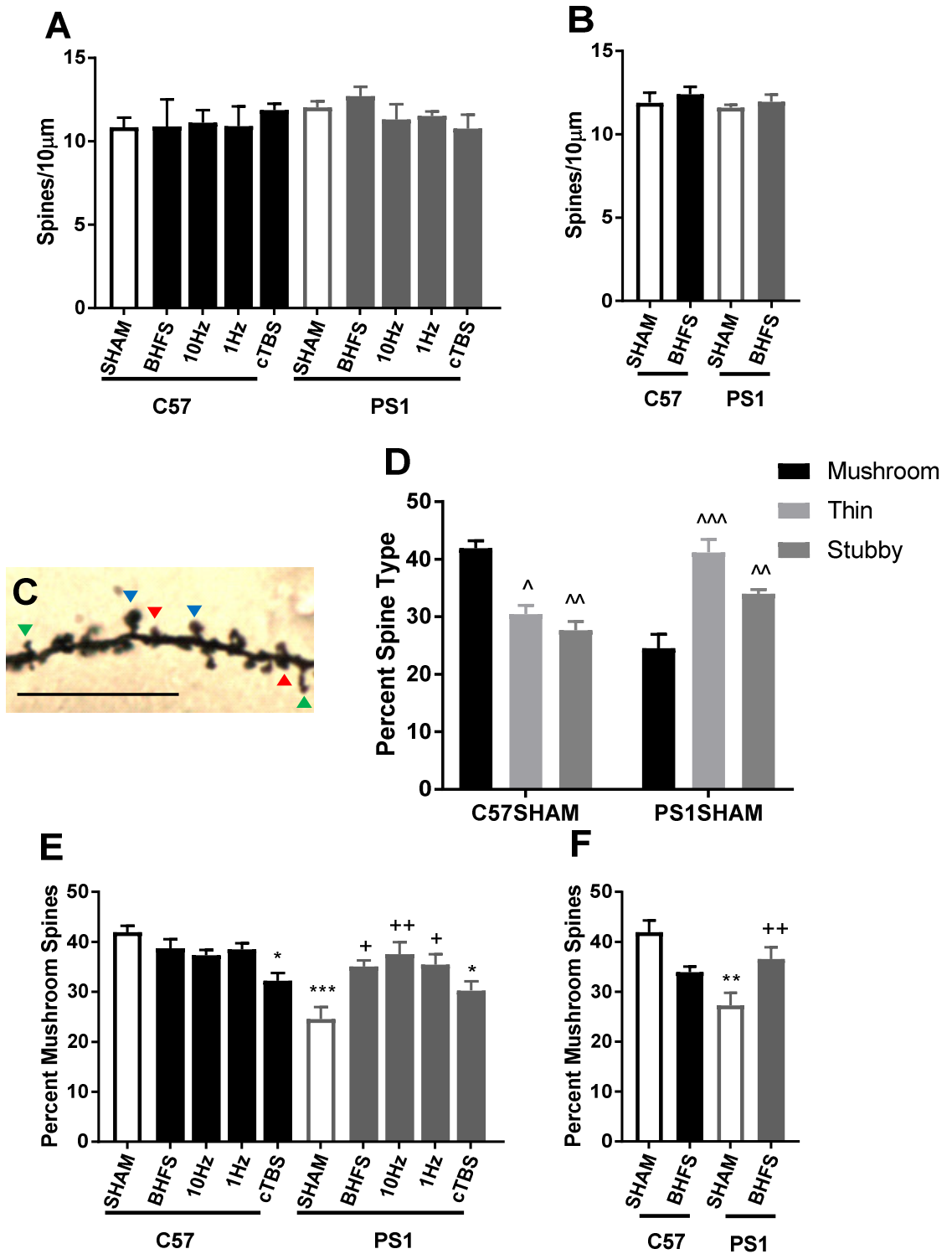


Figure 3.6 LI-rTMS improves dendritic spine abnormalities in PS1M146V mice. Golgi staining was used to visualise dendritic spines in the apical region of the CA1. When assessing the total number of spines there were no differences in the density of spines between groups (A,B). Each spine was classified into different spine types: Mushroom (blue arrows), Thin (green arrows) and Stubby (red arrows), these different types can be seen on the imaged dendritic spine (C). Focusing on the levels of mushroom spines, BHFS, 10Hz and 1Hz improved the levels in 6 months (E) and at 12 months BHFS improved the deficit (F). \wedge $p < 0.05$ $\wedge\wedge$ $p < 0.01$ $\wedge\wedge\wedge$ $p < 0.001$ vs Mushroom, * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$ vs C57-SHAM, + $p < 0.05$ ++ $p < 0.01$ vs PS1-SHAM. Scale bar 10µm.

in PS1 (PS1-SHAM v PS1-BHFS $p < 0.05$, PS1-SHAM v PS1-10Hz $p < 0.01$, PS1-SHAM v PS1-1Hz, $p < 0.05$) mice to a similar level to that of C57 groups (C57-Sham v PS1-BHFS, PS1-10Hz & PS1-1Hz, all $p > 0.05$). Also, while in the PS1 animals, cTBS stimulation did not alter mushroom spine percentage (PS1-SHAM v PS1-cTBS $p > 0.05$, C57-SHAM v PS1-cTBS $p < 0.05$), in the C57 mice cTBS reduced the percentage of mushroom spines (C57-SHAM v C57-cTBS $p < 0.01$). The deficit in mushroom spines did not worsen further with age in these mice (6m vs 12m, $F(1,23) = 0.006$ $p = 0.939$) and a similar interaction between treatment and genotype was retained at 12 months ($F(1,12) = 16.026$ $p = 0.002$, Figure 3.6F). The PS1 genotype had a lower percentage of mushroom spines (C57-SHAM v PS1-SHAM $p < 0.01$) which was increased by BHFS stimulation (PS1-SHAM v PS1-BHFS $p < 0.05$). In summary, these results show a positive effect of BHFS, 10Hz or 1Hz LI-rTMS stimulation on improving the levels of mushroom spines which are reduced in both 6 and 12 month PS1 mice. However, cTBS seemed to have a negative effect on the level on these spines in both C57 and PS1 mice.

Neurogenesis

To identify neurogenesis, a component of learning in the hippocampus, which decreases with age and AD pathology (Kempermann et al. 2004; Wang et al. 2004), mice were injected with the proliferation marker EdU at the start of the second week of stimulation. Four weeks later the number of newly generated cells which differentiated into neurons, as indicated by colocalization with the neuronal marker NeuN, were quantified in the dentate gyrus (Figure 3.7A). Overall, we found less EdU+/NeuN+ double-labelled cells in the dentate gyrus in the PS1 animals compared to C57 (Effect of Genotype $F(1,29) = 9.585$ $p = 0.004$, Figure 3.7B). Moreover, independent of the genotype, LI-rTMS affected the amount of neurogenesis (Effect of Treatment $F(4,29) = 9.585$ $p = 0.009$). Animals treated with cTBS had significantly fewer EdU+/NeuN+ labelled cells than the equivalent SHAM mice ($p < 0.05$), whereas BHFS, 10Hz and 1Hz had no apparent effect. In 12 month old mice, overall there was less neurogenesis than in 6 month mice (Effect of Age $F(1,24) = 11.847$ $p = 0.002$) (Figure 3.7C). However, the difference due to genotype was lost and LI-rTMS had no effect on neurogenesis (Figure 3.7C). Neither the genotype or rTMS stimulation affected the percentage of EdU+ cells which differentiated in neurons (NeuN+) (Figure 3.7D,E), suggesting the effects are coming

from a change to proliferation or survival rather than fate determination of the neural stem cells.

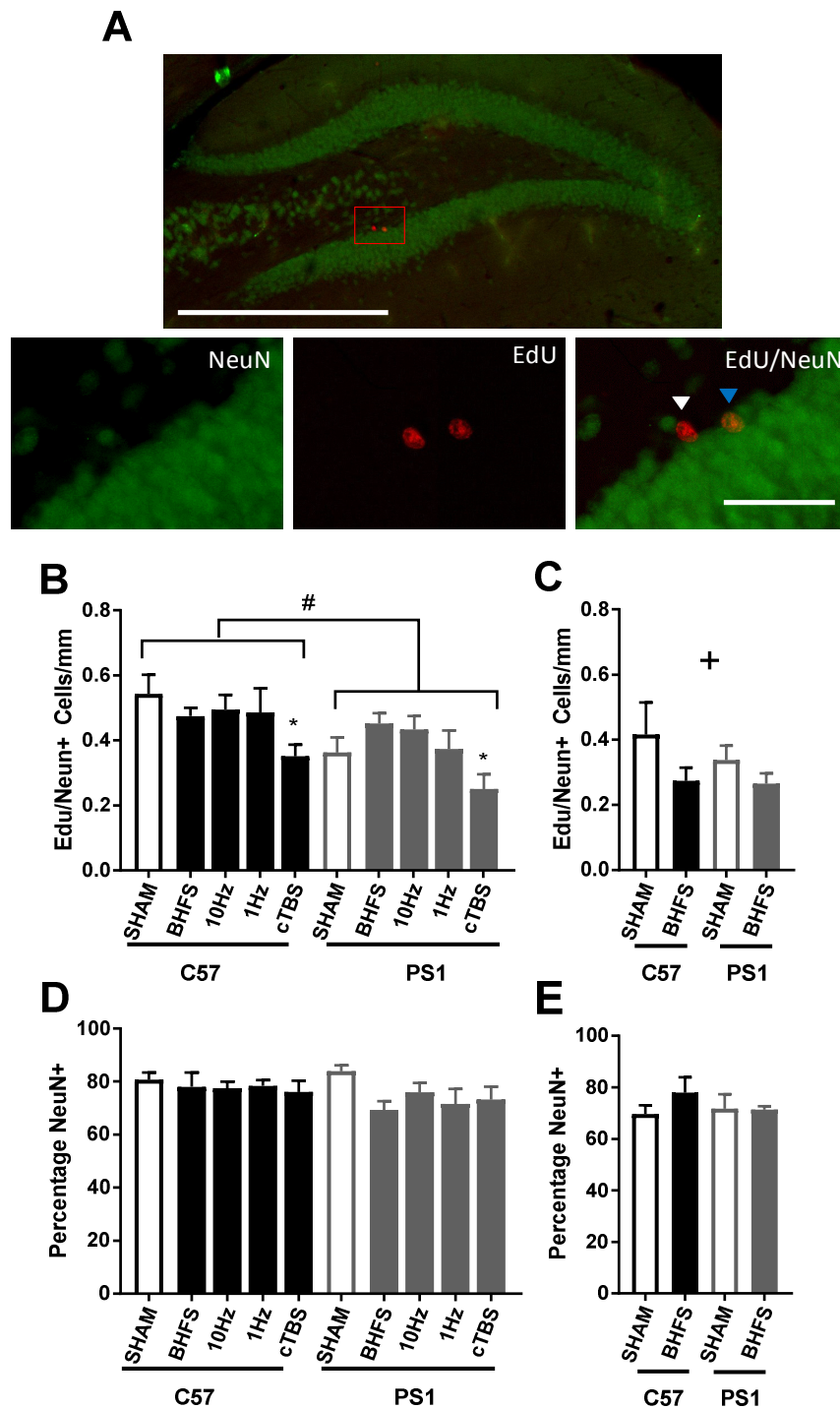


Figure 3.7 Decreased neurogenesis in the PS1M146V mice was not alleviated by LI-rTMS. Proliferated cells were marked with EdU and co-stained with NeuN to classify neuronal differentiation in the dentate gyrus. Representation images of two Edu+ cells with one colocalised with NeuN, indicating differentiation into a granule cell (blue arrow), and one without colocalization (white arrow) (A). The number of proliferated cells was decreased in PS1M146V mice at six months (B), cTBS worsened the neurogenesis in both C57 and PS1 mice. At 12 months there was no difference between groups however over the 12 month group had less neurogenesis than the 6 month group (C, +). There was no difference in the differentiation of neuronal cells between groups (D,E). # $p < 0.05$ Genotype Effect, + $p < 0.05$ Age Effect, * $p < 0.05$ vs PS1/C57-SHAM. Scale bar 500 μ m, 50 μ m.

Hippocampal BDNF

Levels of the neurotrophin BDNF were analysed in the hippocampus (Figure 3.8A,B). BDNF is known to be involved in both the regulation of memory functions and responses to LI-rTMS (Makowiecki et al. 2014; Panja & Bramham 2014; Verpelli et al. 2010). There was no difference in the level of hippocampal BDNF between the C57 and PS1 mice. However, the rTMS stimulation did slightly increase BDNF levels (Effect of Treatment $F(4,28)=2.964$ $p=0.036$). There was an overall frequency effect of the 10Hz stimulation (SHAM v 10Hz, $p<0.05$) however, there were no individual differences between groups.

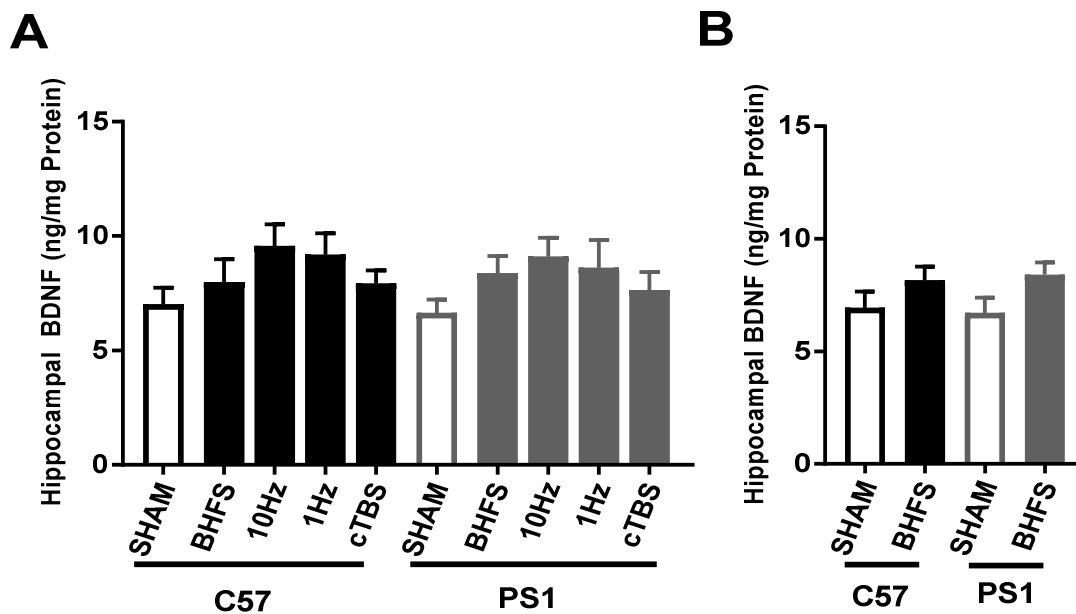


Figure 3.8 LI-rTMS affects hippocampal BDNF independent of genotype. ELISA analysis of total hippocampal BDNF was normalised against the total protein in the sample for both 6 month (A) and 12 month (B) mice. An overall effect of treatment was found in the 6 month animals.

RNA Regulation in the Hippocampus

To better understand the mechanisms underlying the effects of LI-rTMS, we examined hippocampal gene expression changes initiated early in the LI-rTMS regimen (after 3x10 min daily stimulation with BHFS). In comparison with the C57-SHAM control group a total of 10 genes were found to be significantly regulated by either the BHFS stimulation or affected by the PS1 mutation (Figure 3.9). The PS1-SHAM mice had lower expression of *Adora1*, *Alk*, *Apbb1*, *Apoe*, *BDNF*, *Creb1* and *Dlg4* (C57-SHAM v PS1-SHAM $p<0.05$). From gene and phenotype ontology analysis on these seven genes significantly related phenotypes included: Abnormal dendrite morphology, abnormal central nervous system synaptic transmission, increased anxiety-related response, abnormal spatial

learning, decreased vertical activity and allodynia (Supplementary Figure 3.S3,S4). In the PS1 mice, three days of BHFS stimulation significantly improved expression of BDNF and Creb1 (PS1-SHAM v PS1-BHFS, $p < 0.05$), but not Apbb1 and Apoe expression (C57-SHAM v PS1-BHFS, $p < 0.05$). Also, the stimulation modulated gene expression relevant to cell survival and anti-inflammation including: increasing Notch1 expression irrespective of genotype (C57-SHAM v C57-BHFS, C57-SHAM v PS1-BHFS, $p < 0.05$); increasing Bcl2 and decreasing Cxcl1 expression in C57 mice (C57-SHAM v C57-BHFS $p < 0.05$).

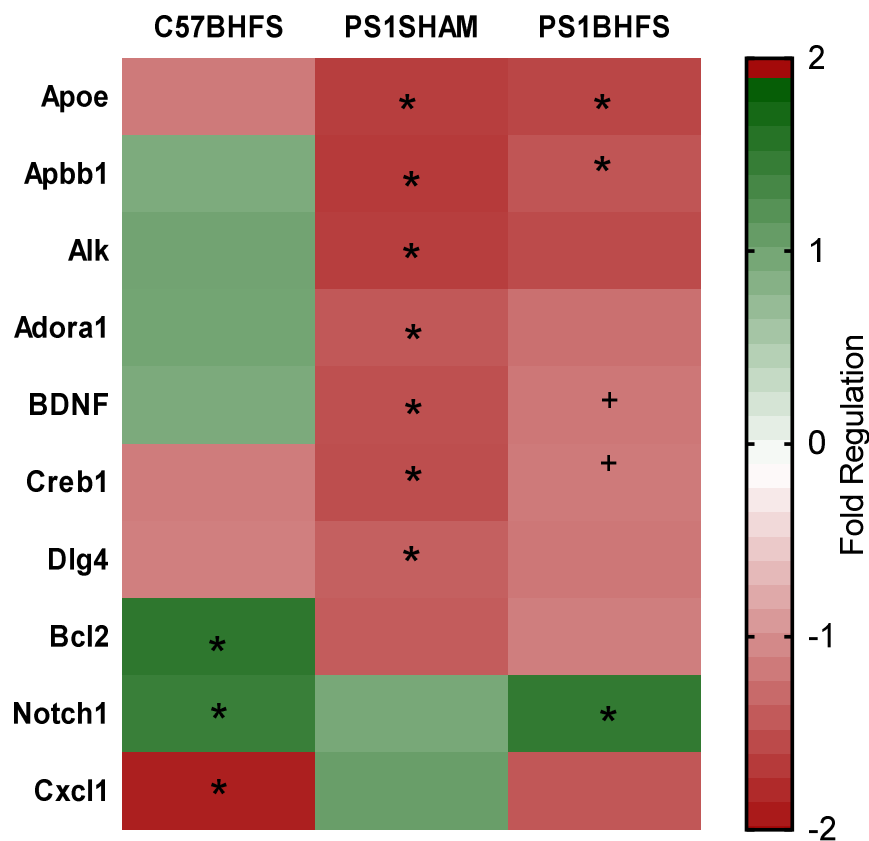


Figure 3.9 Hippocampal gene expression changes after LI-rTMS. RT2 PCR Neurogenesis Array was used to assess the change in specific gene expression after three days of LI-rTMS treatment. Only genes which had a significant change in Fold Regulation are shown in the heat map. * $p < 0.05$ v C57-SHAM, + $p < 0.05$ v PS1-SHAM

Discussion

In this study the potential positive effects of LI-rTMS for treating AD were demonstrated using the PS1M146V animal model of AD. After two weeks of treatment spatial memory deficits and mushroom spine levels were improved using the two excitatory stimulations (BHFS and 10Hz) in the 6 month mice and by using BHFS stimulation in 12 month mice.

This was without improvements in anxiety. In contrast, cTBS had negative effects in the model, while 1Hz showed some improvements but not to the same extent as BHFS and 10Hz.

High frequency hippocampal LI-rTMS improves spatial memory, but not anxiety

After two weeks of excitatory stimulation, LI-rTMS improved the spatial memory deficit in the PS1 mice. Deficits in spatial memory are specific to the AD type of dementia and are exhibited early in the pathology (Bird et al. 2010; Chan et al. 2016; Pengas et al. 2012). As excitatory LI-rTMS specifically improved this memory function from 6 months it demonstrates a potential to be a targeted treatment for early cognitive changes in the disorder. The use of a low intensity form of rTMS distinguishes the results of this study from previous studies investigating rTMS in rodent models of AD (Huang et al. 2017; Tan et al. 2013a; Wang et al. 2015). The small rodent coils used here can target the dorsal hippocampus, which provides a novel method of delivering reasonably focal, low intensity stimulation, the implications of this focality are discussed below.

Moreover, this improved spatial cognition is not due to a simple alleviation of mood. As clinical studies investigating high intensity rTMS in AD often stimulate areas associated with anxiety and depression, there has been difficulty deciphering improvements in cognition from improvements in mood (Ahmed et al. 2012; Rutherford, Gole & Moussavi 2013). Depression and anxiety have a high comorbidity with AD (Teri et al. 1999), however other rodent models of Alzheimer's disease sometimes lack this phenotype (Filali et al. 2012; Lalonde et al. 2004). This was the first study to show an anxious phenotype in the PS1M146V model. Female AD mice have been shown to develop more anxiety than males (Ruan, Bobrovskaya & Zhou 2017), which may have contributed to the effect seen in this study. Future studies should examine anxious phenotypes in both female and male mice to see if this effect is gender specific in the PS1M146V model. Targeting the hippocampus with LI-rTMS appears to solely affect cognition in this study as it had no effect on the anxious phenotype, whilst still improving the spatial memory performance of the PS1 mice. This suggests that LI-rTMS may prove to be an effective targeted treatment for cognition. However, using multisite stimulations to also target areas such as the DLPFC to improve these negative neuropsychological symptoms should not be excluded as they also contribute to worsening quality of life and are inherently hard to treat in these patients (Lyketsos et al. 2003).

LI-rTMS targets specific neurobiological components of learning and memory

Dendritic spine morphology is linked with learning and memory (Kasai et al. 2010). Larger mushroom spines are involved in the accommodation of a greater number of receptors and stabilisation of LTP (Matsuzaki et al. 2001; Yang et al. 2008). PS1 mice have dendritic spine abnormalities, specifically as confirmed by this study, a shift from these stable mushroom spines to thin and stubby types (Sun et al. 2014; Zhang et al. 2015a). A lack of spine stability has been shown to contribute to the spatial memory deficits (Hung 2009) and therefore provides a direct link between phenotype and pathology in this model. We also showed there was a decrease in the regulation of genes related to dendritic spine morphology, including a decrease in expression of Creb1, BDNF and Dlg4 (PSD95). Other studies have also shown decreases in the levels of these proteins separately in the PS1M146V model (Peng et al. 2009; Sun et al. 2014), and though one study found increased Creb and BDNF this analysis was conducted on very young mice (1-3 months) (Shilling et al. 2014) and may be instead reflective of the enhanced signalling state these mice do present at earlier stages which by 6 months develop to an AD-like pathology (Auffret et al. 2010). As it is known that Creb activation causes BDNF transcription (Tao et al. 1998) and BDNF increases the dendritic scaffolding protein, PSD-95 (Hu et al. 2011) these results (reduced expression of Creb1, BDNF and Dlg4) present a theoretical downregulated signalling cascade which could lead to the destabilisation of spine structure.

Excitatory LI-rTMS reversed these dendritic spine abnormalities, after two weeks of stimulation the PS1 mice had the same percentage of mushroom spines as controls. Evidence suggests the stimulation is probably increasing the stability of mushroom spines as previous research showed that LI-rTMS does not induce spine growth in rodents (Sykes, Makowiecki & Rodger 2013). Additionally in PS1 mice, although normal spine growth does occur after an LTP stimulus, the major dysfunction of synaptic spines is an inability to maintain their large structure so these spines return to their original size within hours (Zhang et al. 2015a). Even if the stimulation caused growth of mushroom spines, these spines would not have survived until quantification (after three weeks of behavioural testing). Therefore LI-rTMS must instead be affecting the underlying signalling processes that maintain the structure of already formed mushroom spines.

Similar to previous studies, the changes observed to the PS1 dendritic spines were only seen in the apical dendrites, (Sun et al. 2014), but this was the first study to show a lack of effect of LI-rTMS on the basal dendrites. Apical and basal dendrites are both implicated in spatial memory and learning but the initiation of LTP occurs under different mechanisms in these two dendrite populations (Navakkode et al. 2012; Sajikumar et al. 2007). In apical dendrites LTP has been shown to be reliant on Ca^{2+} /calmodulin-dependent protein kinase II (CAMKII), whereas in basal dendrites Protein Kinase A is involved (Sajikumar, Navakkode & Frey 2007). As already described in the PS1M146V model, CAMKII is decreased (Sun et al. 2014) which would only affect the LTP induction in apical dendrites. Additionally, the LI-rTMS only affected the apical dendrites, improving the loss of mushroom spines. In mouse dorsal hippocampal anatomy, apical dendrites are physically below the basal dendrites, so the differential changes we observed are not simply due to the magnetic field penetration. Calculations of magnetic field strength undertaken in our lab indicate that the magnetic field induced by the small mouse coils extends even to lower hippocampal structures (Makowiecki et al. 2014) thus acting on both apical and basal dendrites. A more likely explanation of the differential effect on the two dendritic fields is consistent with LI-rTMS having greater effects on abnormal, than normal, circuits (Makowiecki et al. 2014; Rodger et al. 2012).

In contrast to its positive effect on dendritic spines, LI-rTMS did not improve the loss of neurogenesis in the PS1 mice, in fact cTBS stimulation worsened neurogenesis. Higher intensity, excitatory forms of rTMS are known to increase neurogenesis (Czéh et al. 2002; Heath et al. 2018; Ueyama et al. 2011). However, we did not see the same effect of excitatory LI-rTMS suggesting increasing neurogenesis requires a threshold level of stimulation (Heath et al. 2018). Additionally, the timing of when the proliferation marker is injected can influence the perceived effect rTMS has on neurogenesis. A previous study, where a proliferation marker was injected either at the start or at the end of rTMS, has shown that the same rTMS protocol can influence cell proliferation and survival differently (Czéh et al. 2002). Therefore, we cannot exclude the idea that we did not capture the correct time point to witness the effect of LI-rTMS as from the PCR results both Notch 1 and Bcl2 were increased after rTMS. These results suggests a potential positive influence of BHFS stimulation on neurogenesis as these genes are involved in survival of proliferated neurons (Oishi et al. 2004; Wang et al. 2009).

Interestingly, in our study both C57 and PS1 mice stimulated with cTBS had less neurogenesis and exhibited worse learning strategies the MWM. Path efficiency during the learning phase in the MWM is related to neurogenesis (Garthe, Behr & Kempermann 2009). However, we cannot conclude from this study whether the performance in the learning tasks was affected by or influenced the levels of neurogenesis. This distinction is particularly important in this study as at the time of performing the MWM the neurons marked by the EdU injection would have begun to integrate into networks, a time when they are very responsive to external influences (Epp et al. 2011; Gu et al. 2012). Therefore, future studies should include groups of animals which either did not perform behavioural tasks or did not have the LI-rTMS, to assess the involvement of these tasks in changing brain plasticity.

LI-rTMS did have a slight effect on increasing levels of BDNF, but not the same extent seen in previous work (Makowiecki et al. 2014). The effect of LI-rTMS on BDNF can be seen acutely after the cessation of stimulation (Makowiecki et al. 2014), whereas in our study BDNF was quantified three weeks after the treatment period. The short term PCR results indicate a potential effect, showing the stimulation did increase BDNF regulation in the PS1 mice after three days. This means the slight long-term changes in the ELISA may be indicative of an earlier BDNF response to rTMS returning to normal levels and suggests the need for repeated treatments to maintain the effects of the stimulation.

LI-rTMS has a unique mechanism of action

The stimulation intensity of the LI-rTMS coils used in this study cannot produce action potentials and therefore have different mechanisms of action compared to high intensity rTMS. The stimulation did not affect amyloid related gene expression, but did influence CREB1, a protein activated by calcium cascades, this supports previous work showing this particular type of stimulation affects calcium release from internal stores within neurons (Grehl et al., 2015). Similar calcium increases within neurons are also seen using other forms of low intensity stimulation such as ELF-EMF (Li et al. 2014b; Luo et al. 2014). Calcium is an important regulator of neuronal functions and is involved in the induction and maintenance of LTP and memory (Brini et al. 2014). Its imbalance has also been implicated in normal ageing, AD and in this particular PS1M146V model, wherein neurons have elevated ER calcium which has been shown to contribute to the dendritic and LTP abnormalities (Kim et al. 2015; Stutzmann et al. 2004). As LI-rTMS

targets calcium release from intracellular stores this may restore calcium homeostasis and decrease the adverse effects of elevated ER calcium: reduced STIM2 expression, reduced activation of CAMKII and elevated calcineurin proteins (Kim, Violette & Ziff 2015; Sun et al. 2014; Zhang et al. 2015a).

Stimulation frequency plays an important role in the efficacy of LI-rTMS. The two “excitatory” frequencies (BHFS and 10Hz) were the most effective in reducing the AD related pathology in the PS1 mice. Whereas the two “inhibitory” frequencies had different effects. 1Hz had some positive effects but not to the same extent as the excitatory frequencies whereas cTBS stimulation had a negative effect, even in the wildtype C57 mice. It is important to understand the differences in frequency to unravel the mechanisms underlying LI-rTMS. This can be related back to the known effect of LI-rTMS causing ER calcium release (Grehl et al. 2015). Temporal patterns of calcium signalling can activate CAMKII or calcineurin through calmodulin, even in small microdomains these changes are observed (Fujii et al. 2013). CAMKII is thought to contribute to LTP (Giese et al., 1998) and calcineurin to LTD (Zeng et al., 2001). In previous studies reduced CAMKII and elevated calcineurin has been implicated in both animal models and human patients with AD (Kim, Violette & Ziff 2015; Sun et al. 2014). Therefore, if the excitatory forms of stimulation activated CAMKII this stimulation provides a novel mechanism to influence this therapeutic target and if cTBS is causing activation of calcineurin this may result in an additive effect and cause a worsening pathology in these mice. These results stress the importance of frequency consideration when using rTMS.

Age affects the response to treatment

Finally, we extended our study to include a 12 month group to assess the effect of advancing AD pathology on both the phenotype and treatment with LI-rTMS. The positive effect of the BHFS stimulation extended to this cohort. This shows that LI-rTMS could still have positive effects in a more developed disorder state, however the PS1 model has a slow disease progression and even at 12 months these mice still do not have extremely debilitating deficits (Auffret et al. 2010). Unexpectedly, at 12 month the control mice had worse spatial memory after stimulation. This may be a result of the effects of normal ageing on the neurons. Ageing has been associated with decreased in calcium buffer proteins resulting in a delay of calcium removal (Kumar, Bodhinathan &

Foster 2009; Verkhatsky & Toescu 1998). Therefore, the same mechanism which is helping improve function in the PS1 mice by relieving the elevated ER calcium may in itself be causing calcium dysregulation in unaffected aged neurons.

Stimulation of other targets

As the target of stimulation for this study, the dorsal hippocampus, is a deep brain structure, more superficial areas such as the cortex would also be affected by the stimulation. We cannot rule out an effect of the stimulation on these cortical areas, which encompass somatosensory, visual and parietal cortices (Lein et al. 2007), contributing to the improved behaviour observed. Currently, research in the PS1M146V model has not explored the effect of the mutation on the cortex. However, because the somatosensory and visual cortices are relatively unaffected until late stages of AD (Frisoni et al. 2009), it would be surprising if these mice were to have deficits in these areas. In addition, in C57 mice the visual pathways and cortex are normal at 12 months of age (Yang et al. 2018) and in the somatosensory cortex there is synaptic loss, particularly of inhibitory synapses, but only by 24 months (Cali et al. 2018), therefore the age groups used in this study could be expected to have normal visual and somatosensory processing. This is confirmed by our control experiment of the Morris Water Maze cue task, which assessed for differences amongst groups in their ability to perform a visuo-spatial swimming task and would have identified abnormal behaviours. Moreover, previous research on the effects of LI-rTMS, have shown little to no effect of the stimulation on normal circuitry (Makowiecki et al. 2014; Poh et al. 2018). Therefore, it may be assumed that if these cortical areas are unaffected in this model, any additional effect from the LI-rTMS would be minimal.

One area of interest though is the parietal cortex; in human rTMS studies this has recently been identified as a potential target for rTMS treatment for improving cognition (Wang et al. 2014b). Future studies could explore the effects of this cortical area in the PS1M146V model and subsequent effects of LI-rTMS on these areas.

Conclusion

This study provides evidence that alternative forms of brain stimulation such as LI-rTMS could be potential treatment for AD. By affecting novel targets such as calcium dysregulation this form of stimulation could serve to alter the changes which lead to

synaptic dysfunction. However, further research is needed to draw more direct links between mechanisms of action of the stimulation and the pathological changes in this and potentially other models of AD. These results definitely provide a rationale for continued research into using LI-rTMS and its effects on cognitive decline in AD.

Supplementary Data

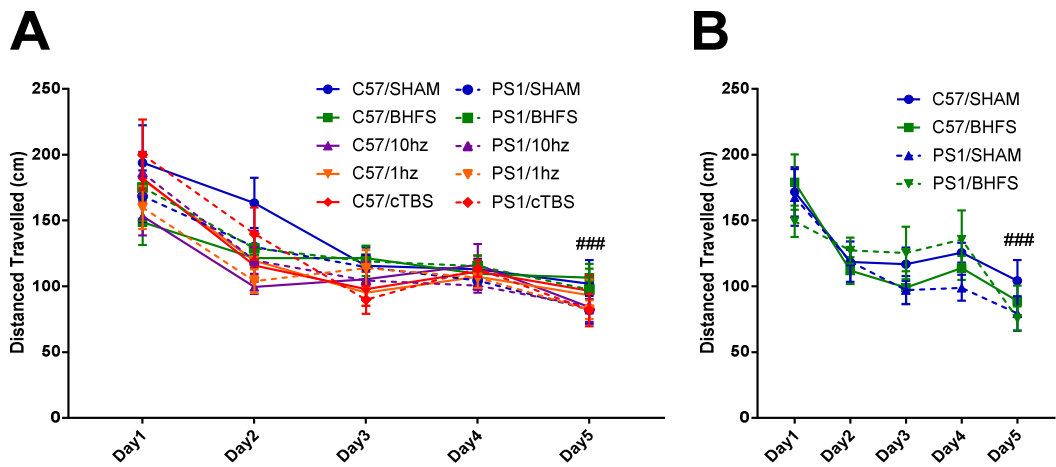


Figure 3.S1 The mice performed equally on the Morris Water Maze Cue Task. All mice easily learnt the cued task over the five days at both 6 (A) and 12 months (B), demonstrating there was no deficit in an ability to perform a Morris Water Maze task. ### p<0.001 vs Day 1

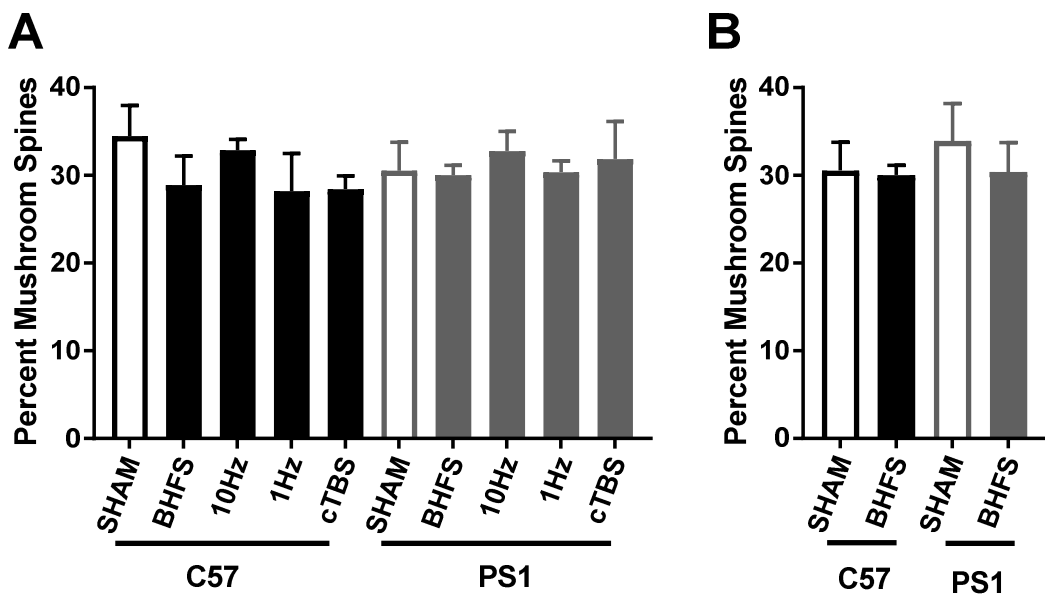


Figure 3.S2 Mushroom spines percentage in basal dendrites. Golgi staining was used quantified number and spine type in the basal CA1 dendrites. There was no difference in the level of mushroom spine amongst 6 (A) and 12 month (B) groups.

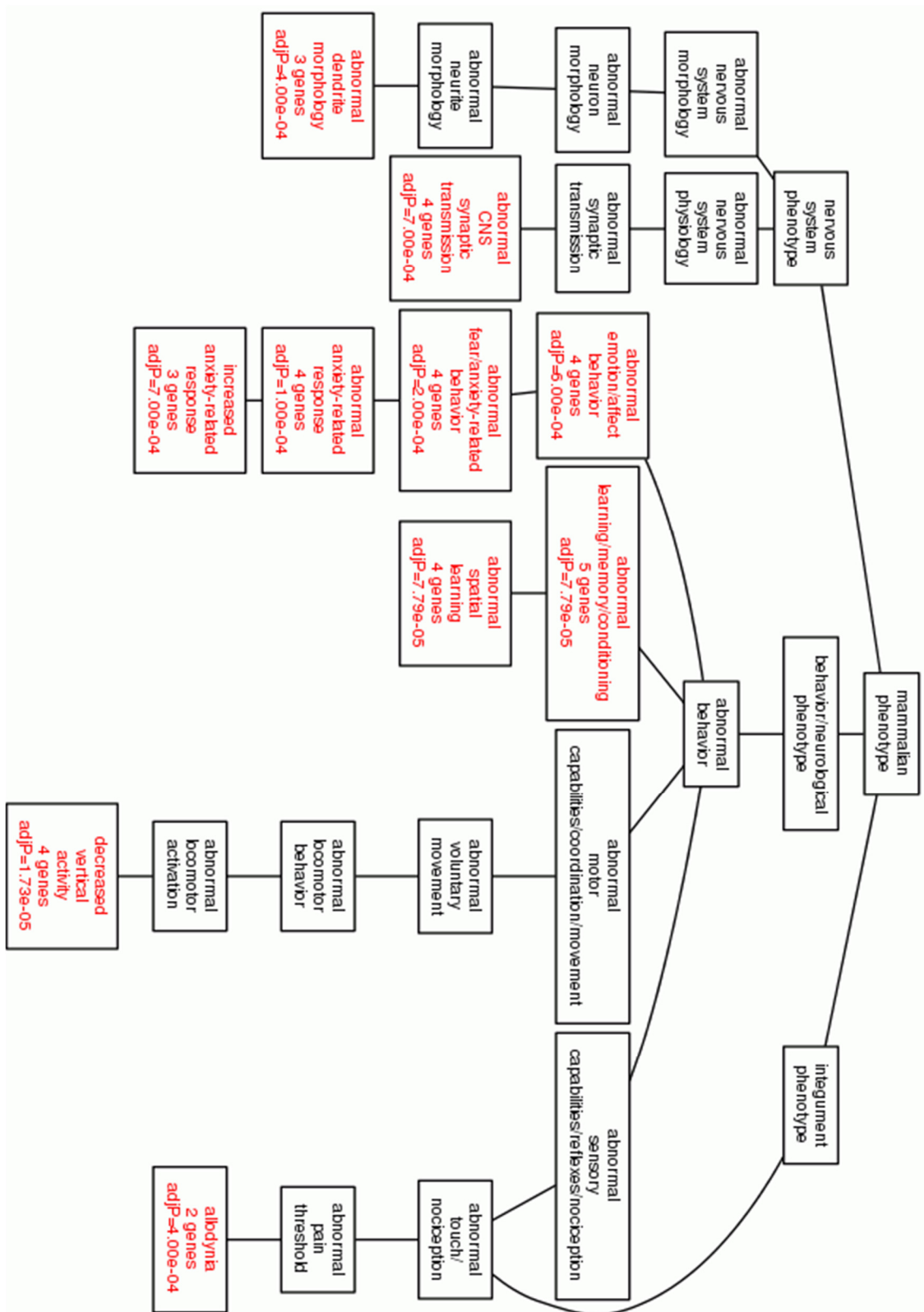


Figure 3.S4 Directed Acyclic Graph of significant phenotype categories related to downregulated genes in PS1 mice. Adora1, Alk, Apbb1, Apoe, BDNF, Creb1 and Dgl4 were analysed using an enrichment analysis for common mammalian phenotypes using Webgestalt 2013.

Chapter 4:

Discussion

This thesis provides novel evidence supporting the use of rTMS to treat different neurological disorders. By examining rTMS in preclinical models of both depression and Alzheimer’s disease, our aim was to investigate how altering the parameters of the stimulation would affect outcomes and inform how to tailor the treatment for each of these disorders. For a summary of main effects of the models used and subsequent treatments from Chapter 2 and Chapter 3, see Table 4.1a and 4.1b respectively.

Table 4.1 Summary of main results from Chapter 2 and Chapter 3. Table 4.1A shows the deficits of the OB model and the three different intensities used (LI-rTMS, MI-rTMS and HI-rTMS) on improving these changes. Table 4.1B highlights the effect of the mutation (PS1M146V) and subsequent treatment with different LI-rTMS frequencies (BHFS, 10Hz, 1Hz and cTBS) at 6 months. For gene regulation analysis only the BHFS frequency was given. ↑ indicates an increase or improvement and ↓ indicates a decrease or worsening of the measure.

A. Chapter 2: OB and Different Intensity rTMS

	OB	LI-rTMS	MI-rTMS	HI-rTMS
Forced Swim Test Immobility	↓	-	↑	↑
BDNF	-	-	↑	-
Neurogenesis	-	-	↑	-
IPA Analysis: Glutamate and GABA functions	↓	-	↑	↑
Corticosterone	-	-	-	↑

B. Chapter 3: PS1 and Different Frequency LI-rTMS

	PS1	BHFS	10Hz	1Hz	cTBS
Elevated Plus Maze	↓	-	-	-	-
MWM Learning	-	-	-	-	↓
MWM Memory	↓	↑	↑	-	-
Neurogenesis	↓	-	-	-	↓
Mushroom Spines	↓	↑	↑	↑	-
Synaptic Plasticity Gene Regulation	↓	↑	N/A	N/A	N/A

Altering the parameters of rTMS can influence the effect in preclinical models

rTMS can be an effective treatment for both depression and Alzheimer's disease (Lam et al. 2008; Liao et al. 2015) even though both of these conditions have very different pathologies. The effectiveness of rTMS as a treatment for either condition depends on the potential of its parameters to target the biological mechanisms underlying these disorders. We therefore investigated how altering different stimulation parameters, specifically intensity and frequency, affected behavioural and neurobiological outcomes in models of depression and AD.

Intensity

We found that using different stimulation intensity can induce distinct biological effects that have a partial dose-dependency. Only medium and high intensity rTMS (MI-rTMS and HI-rTMS), but not low intensity rTMS (LI-rTMS), reduced the psychomotor agitation in the OB model of depression. The antidepressant effect of higher intensity stimulation is likely related to an increased output from the targeted area because HI-rTMS directly induces action potentials and MI-rTMS increases the firing rate of neurons of the area stimulated (Huerta & Volpe 2009; Tang et al. 2016a). Both intensities are therefore similar to those used in human rTMS (HI-rTMS was designed to be equivalent to human rTMS) confirming that outputs of the frontal cortex are important in mediating antidepressant effects (Baeken & De Raedt 2011). Similarly, rTMS to the DLPFC in humans with depression has been shown to cause direct changes to connectivity in the limbic system, as well as regional blood flow changes to various limbic structures such as the cingulate gyrus, hippocampus, amygdala, and thalamus (Speer et al. 2000). Whether MI-rTMS or HI-rTMS is more effective in treating depression was not identified from this study as both improved the behavioural deficit equally. Further research is definitely needed; from human studies a greater antidepressant effect is seen after higher intensities of rTMS (Padberg et al. 2002). However a medium intensity equivalent in humans could allow the delivery of longer pulse trains (as in this thesis) with which complex frequencies can contribute to a greater antidepressant effect (Li et al. 2014a). In comparison, LI-rTMS was not effective in the depression model, but improved memory in the AD model. LI-rTMS delivers subthreshold stimulation that may recruit

metaplastic mechanisms, thus effects are more likely to be limited to populations of neurons directly targeted by the stimulation. The effect of LI-rTMS may thus be highly focal as field measurements from our research has shown that the magnetic field drops off rapidly with increasing distance from the coil, reaching extremely low mT (<2mT) within a few mm, resulting in little stimulation on surrounding brain tissue. This is consistent with our findings in AD where LI-rTMS improved a phenotype associated with plasticity deficits (Auffret et al. 2010) in the targeted hippocampal area. By contrast, in depression where antidepressant effects have been linked to changes in downstream connectivity this low intensity stimulation had no effect.

The manipulation of intensity may therefore be a feasible approach for tailoring treatments to the desired outcome. If influencing network activity is needed, high intensity forms of stimulation may be more effective. However, where there is a need to alter the ability for plasticity to occur in a specific brain region, low intensity stimulation may be better suited.

Frequency

Frequency of stimulation remains a significant focus of rTMS research. For HI-rTMS it is known that high frequency stimulation induces LTP like mechanisms and low frequency stimulation induces LTD like mechanisms (Hallett 2007). From this thesis we have also shown that frequency can be used to induce positive or negative effects even at low intensities. In our AD model we showed positive effects of the “excitatory” stimulations, 10Hz and BHFS, for improving spatial memory and dendritic spine abnormalities, whereas the “inhibitory” stimulations had different effects, 1Hz had some positive effects but not to the same extent as the excitatory stimulations, whereas cTBS had a negative effect on several parameters in both wildtype and PS1M146V AD mice.

In high intensity forms of the stimulation, frequency effects are decoded to initiate LTP or LTD through endogenous brain mechanisms that process information from firing patterns (Fujii et al. 2013). The involvement of these mechanisms in the frequency dependent effects of LI-rTMS are less understood as the stimulation is subthreshold and does not evoke action potentials. Nonetheless, evidence from this thesis suggests that LI-rTMS can still induce frequency dependent effects. In dendritic spines it has been shown that high frequency inputs, which cause calcium influx from the extracellular space, activate CAMKII which initiates LTP; in contrast, low frequency activates

calcineurin which contributes to LTD (Eshete & Fields 2001; Fujii et al. 2013; Giese et al. 1998; Zeng et al. 2001). It is possible that LI-rTMS induced calcium release from the ER (Grehl et al. 2015), even though it is of a smaller magnitude than calcium influx from external sources (Sun et al. 2016), could also differentially activate CAMKII or calcineurin depending on its frequency, and therefore contribute to LTP or LTD without direct depolarisation. A small number of studies have shown that calcium signals from the ER can activate CAMKII or calcineurin, supporting this hypothesis (Mehta et al. 2014; Shakiryanova et al. 2007). Additionally in support of this, in chapter 3 we showed that Creb1, which is activated by CAMKII (Yan et al. 2016), was upregulated after excitatory LI-rTMS. Thus, rTMS frequency, even at stimulation intensities which are subthreshold for inducing action potentials, may be able to be tailored to cause facilitatory or inhibitory effects in a disease context. Understanding the mechanisms of frequency detection by neurons will improve the treatment efficacy.

Summary – rTMS parameters

This thesis has tested specific aspects of rTMS and showed that they can influence outcomes in preclinical models of both depression and AD. Future studies should also investigate other treatment parameters such as days of treatment, pulses per session and even the inter-train interval which have all been shown to be important for determining outcome (Schulze et al. 2018; Volz et al. 2013). This will build upon the knowledge from this thesis for a better understanding of how different parameters of rTMS can influence the outcome in disorder states.

Animal models are essential to assess specific aspects of neurological conditions

The results from this thesis highlight the relevance and usefulness of animal models for investigating specific aspects of neurological conditions. The first study investigated the OB model of depression and revealed that the major behavioural symptom of psychomotor agitation did not respond to antidepressants, but improved after rTMS. These findings suggest that the OB model may be a model of treatment resistant depression (TRD) in which the neurobiological changes involved in treatment resistance can be investigated. In the second study, the PS1M146V AD model is particularly relevant as it reflects the progression of Alzheimer's disease with age, and importantly

presents non-cognitive neuropsychological symptoms associated with the disorder (such as anxiety) that are often not investigated in other animal models of AD.

OB model of agitated treatment resistant depression

The findings in Chapter 2 suggest that the OB model of depression can be used to study aspects of agitated, treatment resistant depression in humans. Currently, studies examining rTMS treatment in animal models of depression often use an pharmacological antidepressant treatment as a benchmark for the effectiveness of rTMS (Feng et al. 2012; Sachdev et al. 2002; Sun et al. 2011), but in humans, rTMS is only used in cases where patients do not respond to antidepressants (Holtzheimer & Mayberg 2012).

We were able to replicate previous findings that OB decreases immobility of mice in the FST (Linge, Pazos & Diaz 2013; Mucignat-Caretta, Bondi & Caretta 2004; Mucignat-Caretta, Bondi & Caretta 2006). This psychomotor agitation was only alleviated by treatment with rTMS but not with the antidepressant fluoxetine. However, we confirmed that fluoxetine was having an effect because it increased serotonin levels in the frontal cortex. Interestingly, psychomotor agitation is one of the major symptoms of the melancholic subtype of depression and this particular subtype shows greater treatment resistance than other forms of depression (Benazzi 2004; Fava 2003). This is the first study to look at the effect of this specific antidepressant on FST psychomotor agitation in the OB model, and it will be important in future to study the effect of other types of antidepressants with different mechanisms of action, such as imipramine which has been shown to have a greater effect in patients with psychomotor agitation (Delini-Stula et al. 1995). In addition, we cannot rule out a mouse strain effect: C57Bl6/J mice have shown treatment resistance to fluoxetine in the past (Otmakhova et al. 1992). Other strains, such as RjOrl:SWISS mice, have a similar psychomotor agitation in the FST after OB (Mucignat-Caretta, Bondi & Caretta 2006), and could be used to investigate if this treatment resistance is consistent across different genetic backgrounds.

Because of the lack of objective measures for diagnosing depression and evaluating treatments in patients it is topical to look at blood biomarkers as a clinical tool. We identified serum AABA and 3-Methylhistidine which were both affected by the OB model and subsequent treatment. AABA has already been found as clinically relevant biomarker for responsiveness to antidepressants (Woo 2015), and our results suggest it could also be a potential biomarker for future studies looking at rTMS treatment.

These results show that the OB model of depression can potentially be used to better understand the neurobiology of treatment resistant depression. As rTMS is an effective alternative treatment in TRD, the OB model could also be used to better understand how rTMS induces an antidepressant effect and eventually this knowledge may help those who have very few options for therapy.

PS1M146V model of Alzheimer's disease

The PS1M146V knock-in model is an established model of AD and this thesis confirmed and expanded existing knowledge of the behaviour and neurobiological changes in this model.

Our behavioural results suggest the PS1M146V model reflects a progressing pathology of AD that also presents a comorbidity of both cognitive and the negative neuropsychological symptoms, which are associated with the disorder in humans. Similar to previous studies we showed that from 6 months of age the PS1M146V mice exhibit worsened spatial memory in the Morris Water Maze (Sun et al. 2005). At 6 months the genotype did not affect learning, however, at 12 months PS1M146V mice exhibited worse search strategies at the beginning of the learning phase. Because memory loss in AD worsens with the progression of the disorder (Gold & Budson 2008), it is important that models of the disease reflect these worsening symptoms. This learning effect has not been detected in this model before and may have been revealed by our examination of subtle differences in swimming patterns (Gehring et al. 2015). PS1M146V mice also displayed an anxious phenotype. Anxiety has a high comorbidity with AD and may be important for contributing to some of the cognitive dysfunction (Teri et al. 1999), therefore it is important that animal models reflect this symptom. The development of anxiety in animal models of AD is contentious; some studies show anxious phenotypes, others show no effect or even a decrease in anxiety (Beauquis et al. 2014; Jensen et al. 2005; Lalonde, Kim & Fukuchi 2004; Lok et al. 2013). Therefore, the presence of anxiety phenotypes in the PS1M146V mice mean that they can be considered good models of the secondary symptoms of AD.

As the hippocampus is central in the pathology of AD it is important to examine changes to its structure and function in models of the condition. In line with previous research we showed that PS1M146V mice have decreased mushroom and increased thin and stubby spines on the apical dendrites of the CA1 neurons, and decreased neurogenesis

in the dentate gyrus (Sun et al. 2014; Wang et al. 2004). Morphological changes in dendritic spines are related to learning and memory (Kopec et al. 2006; Yang et al. 2008). A decrease in the proportion of mushroom spines is thought to reflect a worsened ability to retain memories and from post-mortem studies this decrease has been confirmed in AD pathology (Mahmmoud et al. 2015; Scheff et al. 2006). The decrease in neurogenesis is most likely due to an overall decrease in proliferation (Wang et al. 2004) and ours is the first study to show that this symptom worsens with age in the PS1M146V model. However, as the behavioural tests were conducted during the period when the proliferated neurons were integrating into the hippocampal circuitry, we cannot rule out that the act of learning could have influenced the survival of newly born neurons (Epp, Haack & Galea 2011; Gu et al. 2012). Future testing should include separate groups of animals that did not undergo behavioural testing, to examine the effect learning has on the measures analysed in the PS1M146V mice.

In summary, our results support the idea that the P1M146V model of AD reflects specific and important components of AD pathology. Importantly, the model shows developmental progression of some measures, including the weakening of spatial learning and neurogenesis. Thus, this model has the potential to be useful for research into treatments that can target AD at specific stages, with the aim of slowing progression. This approach is particularly relevant for rTMS because the stage of the disorder has been shown to influence the efficacy of clinical intervention (Ahmed et al. 2012; Padala et al. 2018).

Comparison of animal models

OB and PS1M146V mice each model an underlying cause of their associated disorders: depression and AD respectively. . For the OB model this involved surgical removal of the olfactory bulbs to induce downstream neurological changes and for the M146V model this involved knocking in a specific AD-causing mutation. However, both models have limitations in that they reflect only specific aspects of the complex pathology. In this thesis they have been useful to reflect biological deficits related to these changes, be that the relationship of psychomotor agitation to treatment resistance of depression, or a progressing pathology of AD. Importantly, both these models are essential for researching the ability to tailor rTMS and other treatments for better outcomes in the clinic.

Interestingly, both studies highlight the potential influence of sex in depression and AD models. We chose to use male mice in the depression study as they display prominent changes after olfactory bulbectomy (Linge, Pazos & Diaz 2013) and are less affected by uncontrollable circumstances such as isolation (Voikar et al. 2005). Incidentally, this may also reflect the psychomotor agitation phenotype in humans which is more prevalent in male populations (Khan et al. 2002b). Alzheimer's disease is more prominent in female populations (Viña & Lloret 2010) therefore we used a female mice for this study. This may also be the reason why we saw an anxious phenotype in this model. Research has shown sex specific reproductive hormonal changes have been linked with higher incidence of both anxiety and AD in females (Altemus 2006; Paganini-Hill & Henderson 1994) and for some preclinical models of AD the development of anxiety phenotypes has been shown to be female-specific (Ruan, Bobrovskaya & Zhou 2017). It is also important to keep in mind that rTMS has been shown to have different effects in males and females (Clarke et al. 2017). Therefore, future studies should employ the use of both genders to investigate these gender specific effects in these models.

Limitations in animal studies of rTMS

From the work in this thesis we have attempted to address some current limitations from rodent TMS studies. By stimulating awake mice in both studies we eliminated the possibility that anaesthesia could have modulated the response to rTMS (Gersner et al. 2011). These mice were habituated to the handling and stimulation before experiments began, and though most of the stimulation was delivered successfully we cannot rule out the influence of occasional movements on the position of the coil. Recently, with the advancements within our laboratory of surgically implanted "coil holds" the possible influence movement was demonstrated. In comparison between two studies, one without the coil holds (Makowiecki et al. 2014) and one with (Poh et al. 2018), the use of the coil holds improved the effect of the original study. This highlights the potential for even greater effects if replicating these results with coil holds to maintain a focal stimulation over the area of interest, this especially may influence the lack of effect seen of the LI-rTMS in Chapter 2.

As discussed earlier, whether MI or HI-rTMS induces a better antidepressant effect is yet to be decided and further research is needed to address this idea. Nonetheless, results from our study highlighted some confounding variables that should be addressed,

especially in rodent models, before continuing research. Although they had similar behavioural effects, only MI-rTMS increased BDNF levels and neurogenesis, whereas HI-rTMS increased corticosterone levels. This is in contrast to humans where rTMS reduces levels of circulating corticosteroids (Cohrs et al. 2001; Evers, Hengst & Pecuch 2001). In this study HI-rTMS did not produce any visible signs of distress from the mice and we attempted to limit the stress on the animals by not restraining them as has been done in previous studies (Sun et al. 2011; Wang et al. 2014a). However, as these mice had increased levels of corticosterone there is a possibility that the wide field of high intensity stimulation could have activated peripheral nerves and caused a stress response. Corticosterone is known to negatively impact on both neurogenesis and BDNF levels (Brummelte & Galea 2010; Schaaf et al. 2000) and therefore any increased stress caused by HI-rTMS could have prevented the known effect rTMS has on increasing neurogenesis (Feng et al. 2012; Ueyama et al. 2011).

Another confounding variable which could have influenced the effectiveness of HI-rTMS was its lack of focality in our rodent model. The focality of the magnetic field can have a significant effect on the outcome of rTMS (Groppa et al. 2012). When stimulating humans, coils focally depolarise or hyperpolarise specific brain regions. However, when translating this stimulation to preclinical models the small size of the rodent brain means that a coil which produces focal stimulation in a human stimulates the entire brain. Although the functional consequence of a large stimulation area will depend on many things including the intensity of the stimulation as well as the position and orientation of neurons, it is important to consider these factors when interpreting the results. Unfortunately, even though it may alter the outcome of the stimulation, this lack of focality is generally ignored in animal studies. From this study we cannot dissociate the lack of focality from the potential stress effect of the HI-rTMS coil. As it is not currently possible to build small (8mm) coils that deliver HI-rTMS, we cannot match intensity and focality in rodent models. However, MI-rTMS could be delivered using a large coil to attempt to see if the lack of effect on measures such as BDNF and neurogenesis is still apparent with a stimulation that is not focal but does not induce a stress response.

Evidence of effective tailored treatments

From our studies investigating the different parameters of intensity and frequency in animal models of neurological disorders, potential effective treatments have been

identified for both depression and Alzheimer's disease. These treatments can be related back to the known pathologies of the conditions and highlight the importance of understanding the mechanisms of different forms of rTMS to have the greatest effect. A high intensity stimulation that improves downstream limbic connections may be most effective in depression, whereas in AD, using rTMS to influence the ability of neurons to maintain connections may be more appropriate in a condition where connections weaken over time.

Depression

In the depression model, both MI- and HI-rTMS 10Hz stimulation targeted to the frontal cortex had antidepressant effects. As the behavioural changes in this model are indicative of TRD, this presents an opportunity to explore some of the mechanisms underlying the changes induced by rTMS for this specific type of depression.

Overall, the theory behind the mechanism of rTMS for TRD is hypothesised to be related to the downstream connectivity of the frontal cortex to areas such as the amygdala (Baeken & De Raedt 2011). These areas have a regulatory role in the HPA axis and their activation by rTMS may decrease the hypercortisolism elements of depression (Baeken & De Raedt 2011). This is supported by the fact that only the higher intensity treatments had an antidepressant effect. More specific to our study, the alleviation of the psychomotor agitation using rTMS can also be related to multiple known mechanisms of the stimulation. It is theorised that psychomotor agitation in depression is caused by increased dopamine, increased serotonergic responsivity, decreased GABA and a hyperactive HPA axis (Lindenmayer 2000) and rTMS has been shown to affect all of these mechanisms (Baeken, Lefaucheur & Van Schuerbeek 2017; Evers, Hengst & Pecuch 2001; Gur et al. 2004). The surprising lack of efficacy of fluoxetine in our study may be explained by modulation of serotonergic responsivity. In patients with agitation symptoms, the administration of a serotonin agonist exacerbated these symptoms (Myers et al. 1994), suggesting that increasing serotonin is not an appropriate treatment for these patients. This could explain why fluoxetine, which inhibits the uptake of serotonin had no effect in our model, while rTMS, which has been shown to reduce sensitivity of serotonin receptors, was effective (Gur et al. 2004). Serotonergic responsiveness should be further explored for a better understanding of both the mechanisms of rTMS but also its relationship to depressive symptoms.

As MI-rTMS and HI-rTMS were both effective in reducing the behavioural abnormalities in this model, differences between the effects of these intensities may shed light on their key mechanisms of action. Because only MI-rTMS increased hippocampal neurogenesis and BDNF levels, possibly due to the increase in corticosterone after HI-rTMS as discussed above, these biological changes may not be related to the immediate antidepressant effects of rTMS in our study. The relationship of hippocampal changes to depression remains unclear and there is ongoing debate of whether they are a cause or a consequence of the condition (Sheline 2011). Additionally, in patients with TRD, it is the amygdala and not the hippocampus that increases in volume in rTMS responders (Furtado et al. 2013). Irradiation studies in rodents have shown neurogenesis is necessary in the mechanisms of antidepressants (Santarelli et al. 2003), however as HI-rTMS did not improve neurogenesis but still had an antidepressant effect this alludes to a potential difference in the mechanism of action between rTMS and antidepressant drugs. Because it is not possible to measure real time neurogenesis in humans, animal models are the best way to investigate the significance of hippocampal neurogenesis in depression and its subsequent treatment, and similar irradiation studies could be performed with rTMS in the OB model of depression to confirm whether this brain function is involved in the antidepressant effect of the stimulation

We aimed to explore diagnostic and treatment biomarkers for rTMS and depression by collecting serum from our mouse model. Our metabolomics analysis revealed similar effects of both MI-rTMS and HI-rTMS for restoring normal levels of glutamatergic and GABAergic processes in the blood that were downregulated after OB surgery. However, the interpretation of biomarker data is difficult, particularly for brain disorders, because of the blood brain barrier. The blood brain barrier allows transport of amino acids, however this diffusion is restricted and highly regulated (Smith 2000). Some studies show that serum markers correlate well with the levels of the metabolite in the brain (Bongiovanni et al. 2010; Chen et al. 2016), other studies have shown there is no relationship (Alfredsson et al. 1988). Nonetheless, consistent with our biomarker results, research into rTMS has already confirmed its effect on glutamate and GABA in both humans (Baeken, Lefaucheur & Van Schuerbeek 2017; Michael et al. 2003), and rodents (Yue, Xiao-lin & Tao 2009). Additionally, other treatments for TRD, such as ketamine rely on stimulating glutamatergic systems. Although ketamine is an NMDA antagonist, its

antidepressant effects are related to activation of other glutamate receptors, specifically increased AMPA receptor signalling (Maeng et al. 2008).

As high intensity rTMS focused over the DLPFC is already prescribed for treatment of depression, future studies could employ MI-rTMS or HI-rTMS and utilise the OB model to examine how different frequencies or length of treatment affects the antidepressant effect and further examine mechanisms of action.

Alzheimer's Disease

In the AD model, excitatory LI-rTMS targeted to the hippocampus improved spatial memory. This was a novel effect as currently only high intensity rTMS is used when trying to improve cognition in AD (Wing et al. 2018). One of the main pathological changes in AD is a lack of ability to make lasting synaptic connections and LI-rTMS may be directly targeting that aspect of the pathology.

Excitatory stimulation over the hippocampus in the M146V mouse model for two weeks improved spatial memory performance in both age groups. However, anxiety was not improved, demonstrating the positive effects of this stimulation protocol may be restricted to cognitive functions. It has been suggested in some studies that rTMS may not be targeting only cognition through stimulation of the DLPFC in patients with AD, and that improvement of associated neuropsychological symptoms, such as depression and anxiety, may contribute to the observed improvements in cognition (Ahmed et al. 2012; Rutherford, Gole & Moussavi 2013). Depression and anxiety are frequently comorbid with AD (Teri et al. 1999) and these negative neuropsychological symptoms do not respond well to conventional treatments (Lyketsos et al. 2003). Therefore potentially, a multifaceted treatment could be envisaged, which combines the MI-rTMS treatment explored in Chapter 2 with LI-rTMS to the hippocampus as described in chapter 3, to try to improve both cognitive and psychological dysfunction.

We showed LI-rTMS also affected structural features associated with enhanced plasticity in the AD model. BHFS and 10Hz stimulation restored the percentage of mushroom spines to normal levels. The loss of dendritic spines in this model has been attributed to calcium dysregulation within neurons, specifically elevated levels of ER calcium (Sun et al. 2014; Zhang et al. 2015a). Large mature mushroom spines are very rich in ER, which is critical for their maintenance (Toresson & Grant 2005). As LI-rTMS causes calcium

release from internal stores, it may normalise ER calcium and return the neuron to homeostasis (Grehl et al. 2015). Other studies have also shown similar effects, after other low intensity stimulations, such as ELF-EMF, on intracellular calcium release (Li et al. 2014b; Luo et al. 2014). For example, one study showed that small changes in membrane voltage, that were not enough to cause depolarisation still caused ER calcium release through IP3Rs (Sun et al. 2016). In contrast to ELF-EMF, LI-rTMS is a more targeted stimulation. Diffuse forms of low intensity brain stimulation have been shown to have different effects on cognition, with some studies showing improvements and others a negative effect (Jadidi et al. 2007; Sakhaie et al. 2017; Zhang et al. 2015c). Being able to target specific structures could overcome these inconsistencies. The need for therapies to target this calcium dysregulation in AD has already been expressed (Wang et al. 2017) and LI-rTMS may provide a means to do so.

In the nearly 40 years of rTMS research, the technique has been shown to be well tolerated by most age groups and rarely is associated with significant side effects in healthy controls (Wassermann 1998). However, in our research we found a negative effect of the BHFS stimulation on the aged 12 month, control mice. Aged neurons have less capacity to buffer calcium (Kumar, Bodhinathan & Foster 2009; Tsai et al. 1997; Verkhratsky & Toescu 1998). Therefore, aged mice might have a prolonged calcium response to LI-rTMS, which may proceed from being facilitatory to detrimental over time. The influence of frequency at different ages was not explored in this thesis and definitely warrants further investigation. Interestingly, similar concerns were raised by Grehl et al. (2015), who found that 10Hz, another excitatory form of stimulation, increased the level of neuronal death *in vitro*. We acknowledge the limitations of *in vitro* systems and mouse models, and the difficulty in translating outcomes between these systems into the human brain. Nonetheless, it will be important to investigate further how differently aged neurons respond to different rTMS parameters.

Conclusion and future direction

This thesis has contributed to the understanding of basic mechanisms of rTMS and in particular how altering the parameters of stimulation can influence these mechanisms. We also applied this knowledge to investigate, in preclinical models, how to tailor rTMS for optimal treatments in depression and Alzheimer's disease, targeting possible neurobiological causes of these conditions. By attempting to translate the effects of

human rTMS in animal models of depression and AD we can now take advantage of these models for fundamental investigations that are impossible in human research or clinical trials. For example, it will be important to elucidate the potential mechanisms behind the difference and similarities of MI and HI-rTMS in depression, how rTMS influences calcium dysregulation in AD, as well as the influence of other rTMS parameters on a range of neurobiological outcomes. This thesis has furthered the knowledge of the efficacy and mechanisms of different rTMS protocols. As neuromodulatory therapies are becoming a more popular treatment avenue for neurological disorders, it is important to continue to optimize this form of non-invasive treatment for the wide range of, as yet untreatable, neuropathologies.

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Appendix A:

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ARTICLE

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Medium- and high-intensity rTMS reduces psychomotor agitation with distinct neurobiologic mechanisms

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Abstract

Definitive data are lacking on the mechanism of action and biomarkers of repetitive transcranial magnetic stimulation (rTMS) for the treatment of depression. Low-intensity rTMS (LI-rTMS) has demonstrated utility in preclinical models of rTMS treatments but the effects of LI-rTMS in murine models of depression are unknown. We examined the behavioral and neurobiologic changes in olfactory bulbectomy (OB) mice with medium-intensity rTMS (MI-rTMS) treatment and fluoxetine hydrochloride. We then compared 10-Hz rTMS sessions for 3 min at intensities (measured at the cortical surface) of 4 mT (LI-rTMS), 50 mT (medium-intensity rTMS [MI-rTMS]), or 1 T (high-intensity rTMS [HI-rTMS]) 5 days per week over 4 weeks in an OB model of agitated depression. Behavioral effects were assessed with forced swim test; neurobiologic effects were assessed with brain levels of 5-hydroxytryptamine, brain-derived neurotrophic factor (BDNF), and neurogenesis. Peripheral metabolomic changes induced by OB and rTMS were monitored through enzyme-linked immunosorbent assay and ultrahigh-resolution liquid chromatography-driven targeted metabolomics evaluated with ingenuity pathway analysis (IPA). MI-rTMS and HI-rTMS attenuated psychomotor agitation but only MI-rTMS increased BDNF and neurogenesis levels. HI-rTMS normalized the plasma concentration of α -amino-*n*-butyric acid and 3-methylhistidine. IPA revealed significant changes in glutamine processing and glutamate signaling in the OB model and following MI-rTMS and HI-rTMS treatment. The present findings suggest that MI-rTMS and HI-rTMS induce differential neurobiologic changes in a mouse model of agitated depression. Further, α -amino-*n*-butyric acid and 3-methylhistidine may have utility as biomarkers to objectively monitor the response to rTMS treatment of depression.

Introduction

Repetitive transcranial magnetic stimulation (rTMS) has been used clinically since 2008 for treatment-resistant major depressive disorder. The virtually infinite parameter space of dosing rTMS (e.g., coil geometry, coil position, focality, intensity, frequency, session length, session number, and brain state) magnifies the challenges of

elucidating mechanisms of action and contributes to diverse clinical outcomes. Notwithstanding their limitations, animal and preclinical models are key tools in refining the mechanistic understanding and optimal stimulus dosing approach for rTMS in major depressive disorder. In many rodent models, the focality of human rTMS cannot be appropriately reproduced because even the smallest animal coil stimulates a large portion of the brain¹. However, coils that achieve focality but at a lower intensity enable the mechanistic study of low-intensity rTMS (LI-rTMS). Low-intensity rTMS may modulate cortical excitability in the frontal cortex of humans, thereby addressing clinical psychiatric symptoms.

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Furthermore, preclinical LI-rTMS protocols may model the perifocal effects of standard clinical rTMS^{2–5}.

The present study examined a mouse olfactory bulbectomy (OB) model to investigate differential effects of rTMS stimulation intensities on depression-related behaviors and neurobiologic characteristics. The OB model was selected because removal of the olfactory bulbs results in modulation of downstream connections with such limbic structures as hippocampus, amygdala, habenular nuclei, and raphe dorsalis, inducing reproducible behavioral changes that are associated with depression^{6,7}. In addition, aspects of these behavioral changes, including hyperactivity or psychomotor agitation in the forced swim test (FST)^{8–10}, anhedonia^{11,12}, neurocognitive impairment¹³, and anxiety¹⁴ reflect the symptoms of agitated depression in humans, which often shows poor responsiveness to pharmacologic treatment^{15–18}. We investigated brain markers of plasticity (i.e., brain-derived neurotrophic factor [BDNF] and hippocampal neurogenesis), brain serotonin, and plasma metabolites in an attempt to better understand how the cellular and molecular effects of rTMS intensity relate to behavioral outcomes.

The study design was motivated by an unmet need for preclinical dose-finding studies of rTMS for treatment-refractory phenotypes and special clinical populations. First, we examined the behavioral and neurobiologic changes in OB mice with medium-intensity rTMS (MI-rTMS) treatment and fluoxetine hydrochloride. We then compared the same outcomes following three different intensities of rTMS to gain insight into relevant neurobiologic mechanisms among variable intensities. Finally, we extended our results by analyzing serum samples for potential biomarkers of depression-like behavior in the OB model and recovery induced by rTMS. We hypothesized that high-intensity rTMS would have the greatest behavioral and neurobiologic impact on an OB model of an agitated, treatment-refractory depression.

Materials and methods

Study overview

To examine behavioral (FST) and frontal cortex 5-hydroxytryptamine (5HT) changes, we first compared mice treated with rTMS, fluoxetine, and their respective controls. These five groups included mice undergoing a sham surgery and receiving sham rTMS ($n = 11$), mice undergoing OB and sham rTMS ($n = 9$), mice undergoing OB and MI-rTMS ($n = 11$), mice undergoing OB treated with fluoxetine ($n = 9$), and mice undergoing OB treated with a vehicle ($n = 9$). 5HT changes were measured in subsets of these mice. In a second set of experiments, mice undergoing a sham surgery and sham rTMS ($n = 11$), OB mice treated with sham rTMS ($n = 12$), OB mice treated with LI-rTMS ($n = 16$), OB mice treated with MI-

Table 1 Overview of experiment 1 and 2 sample sizes

Experiment 1	FST		5HT	
SHAM/SHAM	$n = 11$		$n = 5$	
OB/SHAM	$n = 9$		$n = 3$	
OB/MI-rTMS	$n = 11$		$n = 5$	
OB/Flu	$n = 9$		$n = 4$	
OB/Veh	$n = 9$		$n = 4$	
Experiment 2	FST	BDNF	Neurogenesis	Metabolomics
SHAM/SHAM	$n = 11$	$n = 5$	$n = 4$	$n = 4$
OB/SHAM	$n = 12$	$n = 8$	$n = 4$	$n = 9$
OB/LI-rTMS	$n = 16$	$n = 7$	$n = 7$	$n = 11$
OB/MI-rTMS	$n = 13$	$n = 8$	$n = 5$	$n = 10$
OB/HI-rTMS	$n = 15$	$n = 7$	$n = 6$	$n = 9$

BDNF brain-derived neurotrophic factor, 5HT 5-hydroxytryptamine, Flu fluoxetine, FST forced swim test, HI-rTMS high-intensity repetitive transcranial magnetic stimulation, LI-rTMS low-intensity repetitive transcranial magnetic stimulation, MI-rTMS medium-intensity repetitive transcranial magnetic stimulation, OB olfactory bulbectomy, Veh vehicle

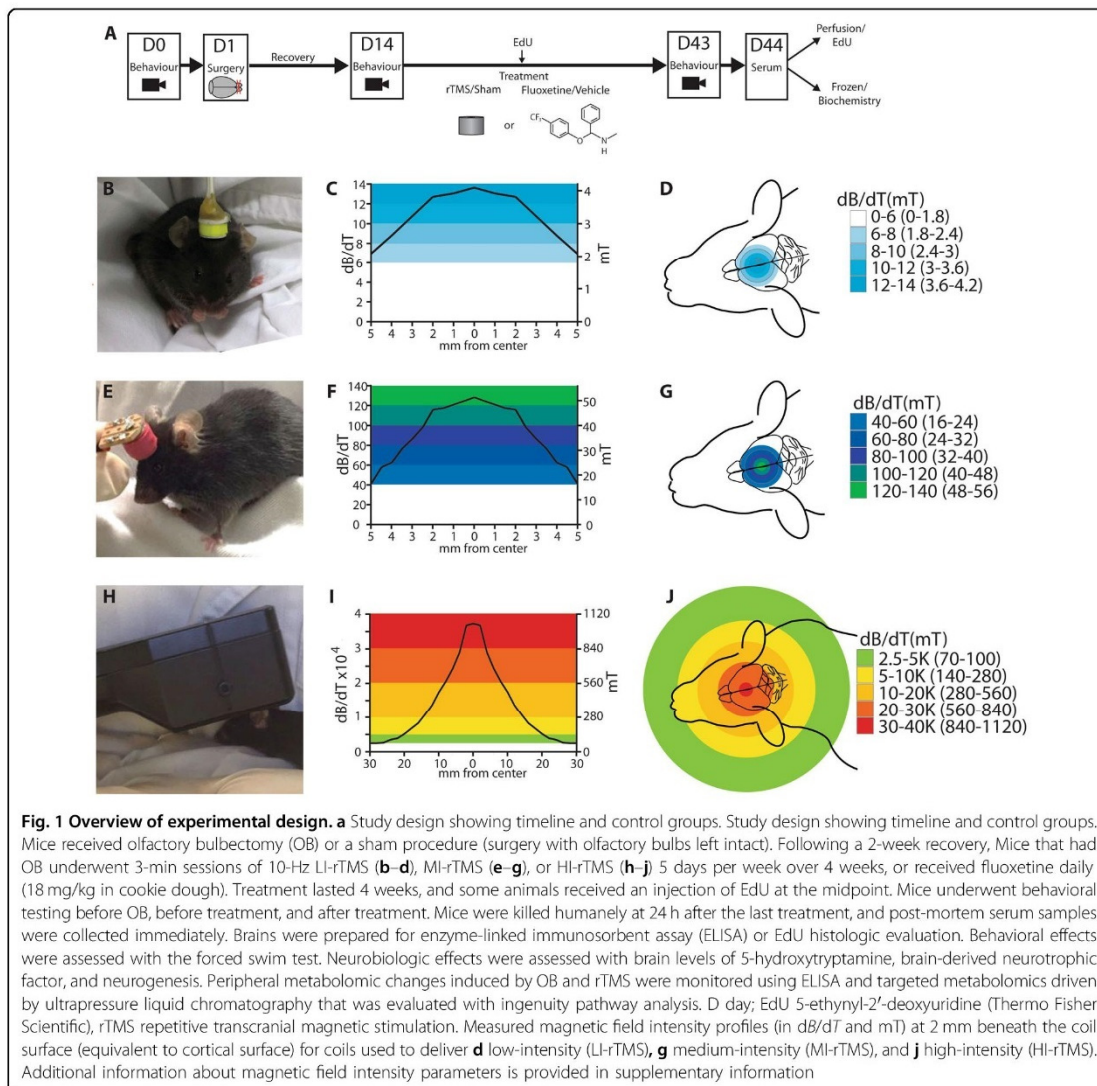
rTMS ($n = 13$), and OB mice treated with HI-rTMS ($n = 15$) had FST testing. Frontal cortex and hippocampal BDNF, hippocampal neurogenesis, and plasma metabolomic studies were conducted in subsets of these treated mice. Table 1 provides an overview of sample sizes. All post-mortem tests and video analyses were blinded.

Study animals

C57BL/6J mice (male sex, aged 8 weeks at start of experiment) were group-housed and maintained under a standard 24-h light-dark cycle (e.g., lights on 0600 and lights off 1800) with ad libitum access to food and water. Each cage contained up to five animals. Mice with aggressive behaviors after OB were moved to separate housing. Housing and bullying were taken into account in statistical analyses. All procedures were approved by the University of Western Australia animal ethics committee (RA03/100/1298) and conformed to US National Institutes of Health guidelines. Experimental design and timeline are shown in Fig. 1a.

Olfactory bulbectomy

OB was performed according to several previously established surgical protocols^{14,19–21}. Briefly, mice were anesthetized with intraperitoneal injection of ketamine (75 mcg/kg; Troy Laboratories Pty Ltd, USA) and medetomidine (1 mg/kg; Troy Laboratories Pty Ltd, USA). A midline incision was made in the skin overlying the skull, and the skin was retracted to reveal the bregma and skull overlying the anterior cranial fossa. Bilateral burr holes



2 mm in diameter were drilled in the skull 4 mm rostral and 2 mm lateral to bregma. The olfactory bulbs were aspirated, and small pieces of hemostatic sponge were used to stop the bleeding before the skin was sutured. Animals receiving a sham operation were treated identically, but the olfactory bulbs were left intact. Following surgery, animals were returned to their home cage. All animals had a 2-week recovery before starting fluoxetine hydrochloride or rTMS treatment. During the second week, all mice were handled daily for 10 min to habituate the animals to the handling associated with rTMS treatment.

Repetitive transcranial magnetic stimulation

rTMS was delivered daily on weekdays for 3 minutes, to deliver 1800 pulses per session, for 4 weeks (Fig. 1a–j). Detailed specifications of the custom coils and stimulators have been published previously^{22,23} and parameters are described briefly herein. For LI-rTMS, a custom-design copper coil (diameter, 8 mm) was connected to a modified E-cell (Global Energy Medicine, Australia). Magnetic field intensity at the coil base was ~12 mT²², and at the cortical surface was 4 mT. For MI-rTMS, a custom-designed air core copper coil (diameter, 8 mm) was controlled by a waveform generator (335141B; Agilent Technologies,

USA) connected to a bipolar programmable power supply (BOP 100-4 M; Kepco Inc, USA). Magnetic field intensity at the coil base was approximately 90 mT²³ and at the cortical surface was 50 mT. For HI-rTMS, a commercially available rat coil (Cool-40 Rat Coil; MagVenture A/S, Denmark) with a 40-mm diameter was used, powered by a MagPro R30 stimulator (MagVenture A/S, Denmark) set to 20% of capable output (Magnetic field intensity coil surface: 1.2 T; cortical surface: 1.0 T). The peak magnetic field adjacent to the coil was measured with a Hall-effect probe (SS94A2D; Honeywell International Inc, USA). Sham-treated animals were handled as if receiving treatment, but the stimulator was not turned on. During treatment, the coil was placed in an anterior position between the ears, overlying the anterior cranial fossa to reliably target the frontal cortex bilaterally (Fig. 1b, e, h). Magnetic field measurements (in dB/dT and mT) for each coil are shown in Fig. 1. A discussion of the magnetic field intensities used in relation to human rTMS parameters is provided in supplemental information (Supplemental Text and Table 1).

Fluoxetine treatment

Fluoxetine (Sigma-Aldrich) 18 mg/kg was administered daily for 28 consecutive days, delivered in portioned balls of cookie dough²⁴. Mice were monitored individually to ensure they had eaten the full quantity. Control mice (called vehicle group) received the same handling and were given a ball of cookie dough without fluoxetine.

Ingenuity pathway analysis

Metabolites identified by targeted metabolomics were uploaded into ingenuity pathway analysis (IPA; Qiagen, Redwood City, CA) for stratification and categorization of direct and indirect network interactions using IPA's functional analysis algorithm and curated IPA ingenuity knowledge base (IPA-KEB). IPA is a web-based software application that allows for the analysis, integration, and interpretation of omics datasets by utilizing known molecular and genetic pathways and established relationships with cellular processes and metabolites. We utilized this integrative database in order to identify pathways and processes affected by bulbectomy and multiple rTMS parameters. Prior to entry into IPA, each dataset of identified metabolites was sorted by Chemical Abstract Service (CAS) number. A metabolomics analysis was carried out using default IPA settings, excluding pathways specific to cancer cell lines. To minimize the incidence of false positive results, expression value threshold filters were set to a 1.25 fold-change ratio between bulbectomized and sham-operated animals with a minimum corrected confidence value of $P \leq 0.05$. The threshold filter for analysis of rTMS treatment was set at 1.5 fold-change ratio between treated and untreated

groups. Using these criteria, IPA was able to generate a reference dataset consisting of all significant and non-significant metabolites identified in sham-treated animals and animals treated with various intensities of rTMS, as well as a focus set of metabolites consisting of those present in significantly different levels than the reference dataset. Biological functions, disease states, and canonical pathways associated with our reference and focus set of metabolites were generated by IPA. The IPA functional analysis generated statistical significances derived from the association of our focus metabolite datasets with molecules already established with biological processes and canonical pathways using a right-tailed Fisher's exact test, where $P \leq 0.05$ was considered significant.

Data analysis

Statistical analysis and graphing were carried out using GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA). Behavioral measures, brain 5HT and BDNF levels, and the number of proliferating cells were analyzed using ANOVA or MANOVA with Sidak post hoc or Dunnett's T. For forced swim test data, we analyzed treatment effects within-subjects, expressed as the difference between post-surgery and post-treatment scores. All data met the homogeneity of error variance assumption, except for BDNF frontal cortex data, which were log transformed and then met assumptions.

The intensity-dependent effects of rTMS on plasma metabolites were analyzed by one-way ANOVA with Tukey's post-hoc analysis. IPA was carried out by averaging the metabolite concentrations under each treatment parameter and comparing these mean values to the average metabolite concentration of the relevant control group in order to calculate the fold-change in metabolite concentration. We also investigated possible correlations between FST performance and BDNF levels, neurogenesis and metabolite levels. The threshold for statistical significance for all experiments was set at $P < 0.05$ except where corrected P -values were used and these cases are indicated in the text.

Power calculations from the first set of behavioral (FST) data including MI-rTMS and fluoxetine demonstrated that $n = 4$ (per group) would provide 0.8 power to detect a 15% differences ($P < 0.05$) in a one-way ANOVA with four pairwise comparisons, with two-sided equality.

All animals underwent behavioral testing and were then randomly allocated to a particular type of post-mortem test. We assumed that the post-mortem tests would be less variable than behavioral tests, so animals were allocated to various tests to ensure that each group size was a minimum of $n = 4$. The variation in group size was due to our efforts to reconcile randomizing animals from different surgery sessions, technical and timing requirements of experiments, a small number of experimental failures,

and attempting to keep group size within experiments as similar as possible. Only one group (OB-Sham for 5HT ELISA) was smaller than 4, but that dataset had low variability, and a subsequent power analysis confirmed that the sample size provided 0.9 power.

Supplemental Information

The Supplemental Appendix describes an estimate of motor thresholds for each rTMS intensity, 5-ethynyl-2'-deoxyuridine (EdU; Thermo Fisher Scientific) labeling of newly born cells, behavioral analysis, and brain and serum collection. The Supplemental Appendix also summarizes serotonin and BDNF enzyme-linked immunosorbent assays, analysis of neurogenesis, and metabolomics.

Results

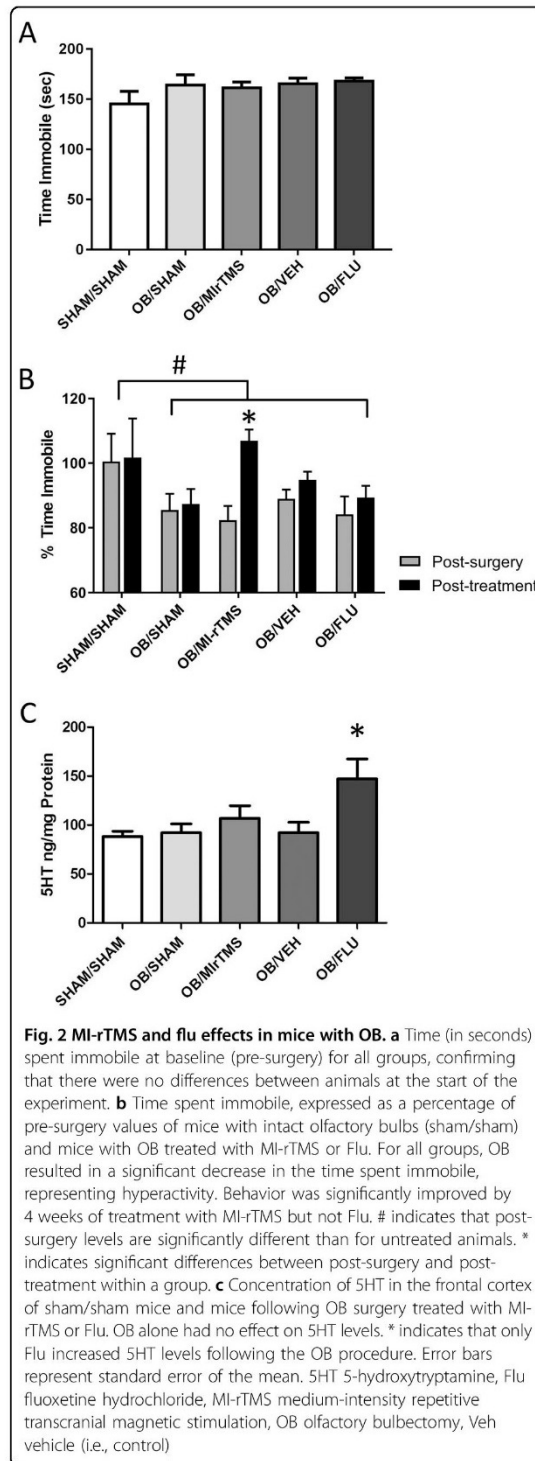
Tolerability

rTMS at all intensities was well tolerated by the mice and there was no sign that the animals felt any sensations during the stimulation and no seizures, freezing, or avoidance behaviors were noted for any of the intensities applied. Furthermore, there were no signs of ongoing pain or discomfort following the stimulation as determined by the facial grimace score²⁵.

Reduction of hyperactivity in the FST by MI-rTMS but not fluoxetine is independent of serotonin levels

As previously reported⁸⁻¹⁰, the FST revealed a significant and reliable decrease in immobility after OB surgery, providing an opportunity to compare the efficacy of rTMS and fluoxetine in restoring normal activity levels. Activity in the FST was not significantly different between groups at baseline (pre-intervention; Fig. 2a) but significantly differed in the change induced by treatment over time (analysis of variance [ANOVA], significant time × group interaction with Greenhouse-Geisser correction for sphericity violation, $F(7.06, 164.91) = 3.702$ ($P = 0.001$)). Follow-up tests for simple effects of time within each group showed a significant decrease in immobility time in the FST from pre-surgery to post-surgery in all groups (P -values < 0.05) except for sham/sham ($P = 0.42$), confirming that OB-induced hyperactivity consistent with a phenotype of agitated depression (Fig. 2b).

To compare the efficacy of MI-rTMS and fluoxetine in reducing OB-induced hyperactivity, we performed further follow-up analyses (Dunnett test), examining rTMS and fluoxetine groups compared with their respective controls and with sham/sham. The MI-rTMS group showed significantly greater improvements between the post-surgery to post-treatment time points in the FST than both OB/sham (Dunnett test, $P = 0.03$) and sham/sham (Dunnett test, $P = 0.02$) (Fig. 2b). However, no significant difference was found between post-surgery and post-treatment for fluoxetine or vehicle groups (Dunnett test, $P > 0.05$) (Fig.



2b). We then investigated whether the behavioral changes were accompanied by a change in 5-hydroxytryptamine (5HT) levels in the frontal cortex at the end of treatment. A significant difference was observed in frontal cortex 5HT levels between groups (ANOVA, $F[4, 38] = 4.113$; $P = 0.007$) (Fig. 2c). Post hoc tests showed that MI-rTMS did not significantly affect 5HT, but the OB/fluoxetine group had significantly increased frontal cortex 5HT levels compared with both vehicle-treated control (Sidak, $P = 0.03$) and sham/sham ($P = 0.004$) (Fig. 2c). Interestingly, the two control groups (OB/vehicle and sham/sham) were not significantly different from each other, suggesting that OB itself did not alter baseline 5HT concentration ($P = 0.86$).

Intensity of rTMS differentially reduces hyperactivity in the FST

We then delivered rTMS at different intensities to determine optimal conditions for reducing hyperactive behavior in the FST. We first confirmed that there were no pre-existing differences in the FST behavior prior to intervention (Fig. 3a). A significant change was observed in immobility time in the FST over time and across groups (ANOVA, $F[2, 144] = 54.64$; $P < 0.001$), indicating that surgery increased activity (agitation) in the FST. All rTMS groups showed some recovery, but only the MI-rTMS ($P = 0.005$) and HI-rTMS ($P = 0.02$) groups improved significantly between post-surgery and post-treatment (Fig. 3b). Confirming our results from the first experiment, none of the rTMS groups showed any change in 5HT levels in the frontal cortex (ANOVA with groups OB-sham, LI-rTMS, MI-rTMS, and HI-rTMS ($F[3, 25] = 1.106$; $P = 0.37$) (data not shown)).

Brain-derived neurotrophic factor

Our initial experiment ruled out a role for 5HT in the effects of MI-rTMS and in the rescue of OB-induced behavior. Therefore, we investigated frontal cortical and hippocampal BDNF levels, as well as hippocampal neurogenesis, as possible mechanisms underlying behavioral change in the cohorts of different rTMS intensity.

ANOVA showed a significant main effect of rTMS treatment on BDNF concentrations in the frontal cortex (ANOVA, $F[4, 29] = 3.368$; $P = 0.02$) and the hippocampus ($F[4, 29] = 2.983$; $P = 0.04$). We followed up by first comparing all groups to the sham/sham group. In the frontal cortex, only the MI-rTMS group had significantly higher BDNF levels than the sham/sham group (Dunnett test, $P = 0.048$) (Fig. 3c). In the hippocampus, BDNF levels were increased to a similar level in the MI-rTMS, HI-rTMS, and OB-sham groups compared with the sham/sham group, although this increase was significant only for MI-rTMS (Dunnett test, $P = 0.04$) (Fig. 3d). No significant differences in BDNF levels were observed

between any rTMS-treated group and the OB-sham group (Dunnett test, $P > 0.05$).

Hippocampal neurogenesis

In a separate cohort, we analyzed cell genesis by injecting EdU at the midpoint of rTMS treatment. The mice were killed humanely 2 weeks later. We compared the density of newly born neurons (EdU and neuron-specific protein [NeuN] double-labeled cells) following the different rTMS intensities (Fig. 3e–g). A significant difference was detected between groups (ANOVA, $F[1, 24] = 2.989$; $P = 0.04$), but only MI-rTMS had a significantly higher number of newly born neurons (Fig. 3e, f, h, i) compared with the sham/sham group (Dunnett test, $P = 0.02$). Further follow-up analyses showed that hippocampal neurogenesis in MI-rTMS was also significantly greater than OB-sham (Dunnett test, $P = 0.02$), but no other groups were significantly different to the OB-sham group (Dunnett test, $P > 0.05$) (Fig. 3e, f, h, i). The percentage of newly born neurons positive for NeuN was not significantly different between groups (data not shown).

Plasma metabolomics

The metabolic effects of OB and the different intensities of rTMS treatment were examined using targeted metabolomics driven by ultrahigh performance liquid chromatography. In total, 6 of the 42 examined metabolites were found to be significantly altered in the plasma of OB mice compared with their sham-treated counterparts. OB decreased the concentration of three bioactive amines— γ -aminobutyric acid (GABA) ($P = 0.049$), AABA ($P = 0.001$), and their molecular precursor glutamine ($P < 0.001$)—as well as β -alanine ($P = 0.048$) and sarcosine ($P = 0.04$). Only 3-methylhistidine was significantly increased by OB ($P < 0.001$) (Fig. 4a). After the OB procedure and rTMS treatment at different intensities, 7 of 42 examined metabolites were determined to significantly interact with rTMS (Supplemental Table 2). However, post hoc analysis revealed that only AABA and 3-methylhistidine were affected by any specific rTMS treatment, being significantly upregulated ($P = 0.004$) and downregulated, respectively, by HI-rTMS ($P = 0.03$) (Fig. 4b). Interestingly, these two metabolites were also significantly altered by OB, and HI-rTMS had the effect of restoring normal levels. All rTMS intensities produced different changes in the plasma metabolic profile, and post hoc analysis revealed that 5 of the 7 metabolites were affected differentially by different stimulation intensities (Fig. 4b).

IPA revealed that the top 4 canonical pathways affected by OB included glutamate-dependent acid resistance ($P = 0.006$), glutamine degradation ($P = 0.009$), glutamine biosynthesis ($P = 0.02$), and GABA receptor signaling (P

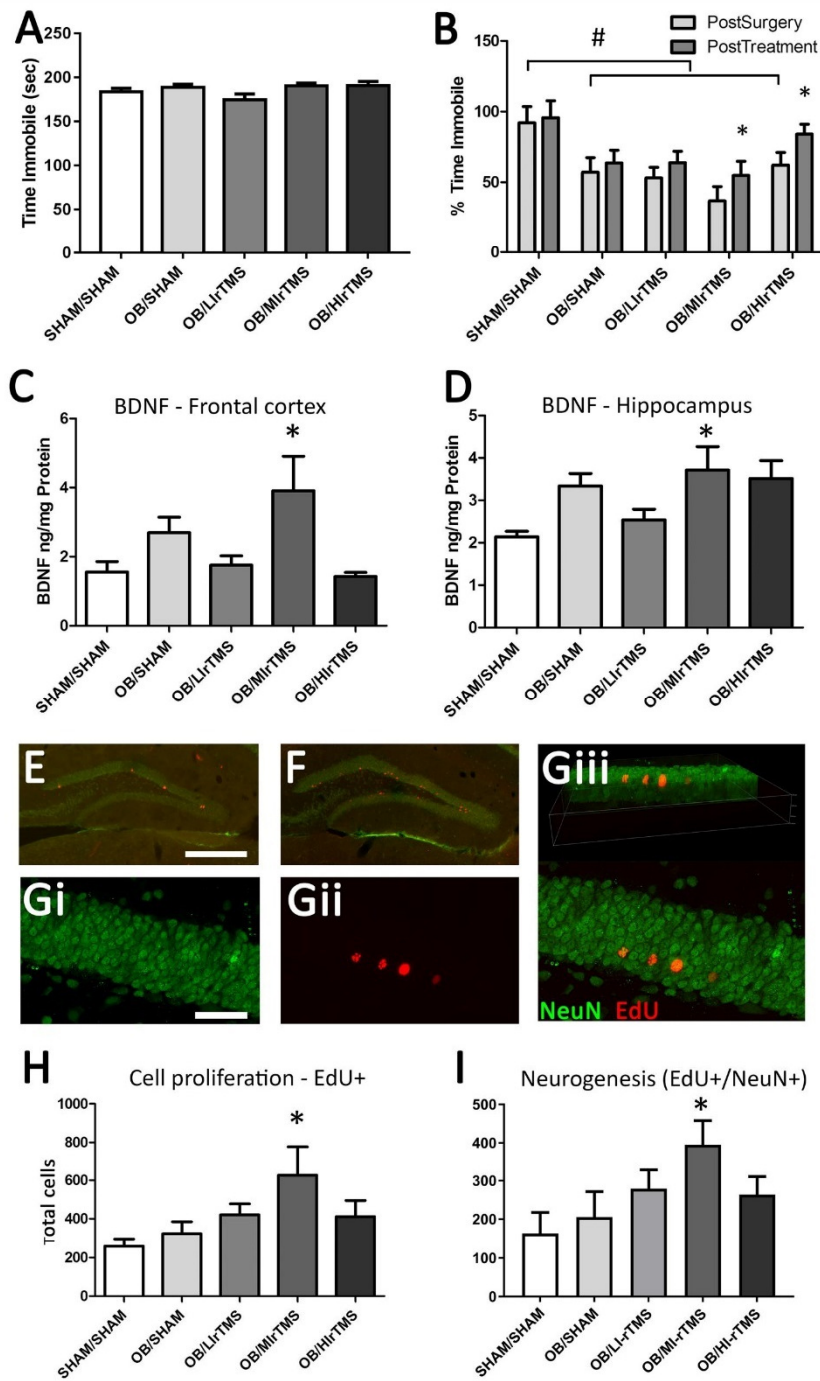
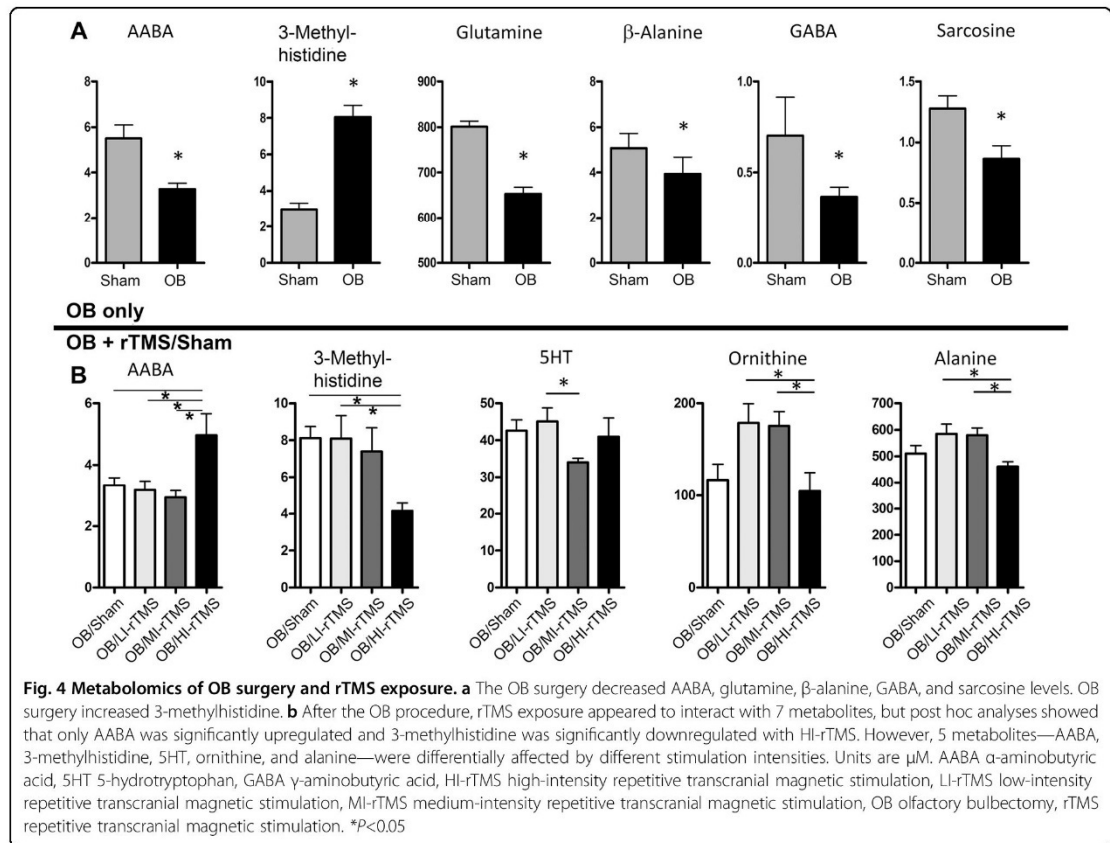


Fig. 3 (See legend on next page.)

Fig. 3 Comparison of rTMS intensities. **a** Time (in seconds) spent immobile at baseline (pre-surgery) for all groups, confirming that there were no differences between animals at the start of the experiment. **b** Time spent immobile in the forced swim test as a percentage of the pre-surgery value for each animal. OB surgery significantly reduced the time spent immobile compared with pre-surgery values, indicating hyperactivity. Behavior was partially rescued by MI-rTMS treatment. # indicates that post-surgery levels are significantly different from untreated animals. * shows significant differences between post-surgery and post-treatment within a group. **c, d** BDNF concentration in the frontal cortex (**c**) and hippocampus (**d**). Only MI-rTMS resulted in increased BDNF levels compared with sham/sham treatment in both frontal cortex and hippocampus, but no significant differences were observed between any groups compared with OB/sham. * indicates a significant difference compared with sham/sham. **e–g** Immunohistochemistry showing EdU single-labeled and EdU-NeuN double-labeled cells in the granular and subgranular layers of the dentate gyrus. **e, f** Low power view of the hippocampus in sham (**e**) and MI-rTMS (**f**)-treated mice showing increased Edu labeling following MI-rTMS. confocal microscopy of MI-rTMS-treated animal confirming co-localization of NeuN (**g**i), EdU (**g**ii), labels merged (**g**iii), with a 3d rotated view showing co-localization of red and green staining within the hippocampal neurons (**g**iii). **h, i** Number of EdU-positive cells (**h**) and EdU-NeuN double-labeled cells (**i**) in hippocampus following OB surgery and rTMS treatment. A significant increase was found in the number of newly born neurons following MI-rTMS compared with OB/sham and sham/sham mice. * shows a significant difference compared with sham/sham and OB/sham. Error bars represent standard error of the mean. Scale bars are 500 μ m (**e**) and 50 μ m (**g**). BDNF brain-derived neurotrophic factor, EdU 5-ethynyl-2'-deoxyuridine (Thermo Fisher Scientific), HI-rTMS high-intensity repetitive transcranial magnetic stimulation, LI-rTMS light-intensity repetitive transcranial magnetic stimulation, MI-rTMS medium-intensity repetitive transcranial magnetic stimulation, NeuN neuron-specific protein, OB olfactory bulbectomy, rTMS repetitive transcranial magnetic stimulation



= 0.02). Glutamate receptor signaling was also significantly affected by OB ($P = 0.02$) (Supplemental Fig. 1).

The IPA results were suggestive that various rTMS stimulation parameters had differential effects on

peripheral metabolites. For example, LI-rTMS altered metabolites associated with glutamate-dependent acid resistance ($P = 0.006$), inducible nitric oxide synthase signaling ($P = 0.009$), the antiproliferative role of the

somatostatin II receptor ($P=0.01$), inhibition of angiogenesis by thrombospondin-1 ($P=0.01$), and neuronal nitric oxide synthase signaling in neurons ($P=0.01$) (Supplemental Fig. 2A). MI-rTMS significantly affected some of the same canonical pathways, such as glutamate-dependent acid resistance ($P<0.001$) and neuronal nitric oxide synthase signaling in neurons ($P<0.001$), but also affected arginine biosynthesis ($P<0.001$), the citrulline metabolism superpathway ($P<0.001$), and aspartate biosynthesis ($P<0.001$) (Supplemental Fig. 2B). LI-rTMS and MI-rTMS also modulated canonical pathways associated with glutamate degradation and GABA receptor signaling (Supplemental Figs. 2A and 2B). Similar to other rTMS stimulation paradigms, HI-rTMS significantly altered glutamate-dependent acid resistance ($P=0.006$) and GABA receptor signaling ($P=0.02$) (Supplemental Fig. 2C). It also upregulated canonical pathways involved in glutamate degradation ($P=0.02$), albeit by altering a different branch of the catabolic pathway. HI-rTMS also significantly altered canonical pathways associated with histamine biosynthesis ($P=0.006$) and ceramide degradation ($P=0.02$). Notably, glutamate-dependent acid resistance, glutamate degradation, and GABA receptor signaling pathways were downregulated by OB and upregulated by all rTMS intensities.

Correlations

When all animals were included in the analysis, FST performance post treatment showed a significant negative correlation with BDNF levels (Frontal cortex: Pearson's correlation -0.365 ; $P=0.034$; Hippocampus: Pearson's correlation -0.359 ; $P=0.037$) and a significant positive correlation AABA (Pearson Correlation 0.474 ; $P=0.029$), but these correlations were not observed within any individual treatment group. When groups were analyzed separately, only animals in the Sham-OB group showed a positive correlation with 3-methyl histidine (Pearson's correlation 0.694 ; $P=0.038$).

Discussion

To our knowledge, this is the first study to suggest that MI-rTMS and HI-rTMS improved hyperactivity in mice with OB, while fluoxetine did not. These findings suggest that OB may be a valid murine model of an agitated, treatment-resistant depression. MI-rTMS-treated mice had increased BDNF levels in the frontal cortex and hippocampus, as well as increased neurogenesis in the hippocampus. None of the rTMS-treated groups had increased serotonin levels. Plasma metabolites such as AABA and 3-methylhistidine, which are involved in glutamate and GABA metabolic signaling pathways, may have utility for further translational research of biomarkers for rTMS.

Similar to the OB model used in the present study, some forms of depression in humans are associated with an

agitated phenotype marked by increased stress reactivity and psychomotor agitation, increased nocturnal activity, and impaired concentration¹⁸. Previous studies of OB in mice have consistently revealed a phenotype of psychomotor agitation^{7–10} and we show for the first time that this behavior is resistant to treatment with fluoxetine, similar to clinical human populations with treatment-resistant depression^{15–18,26}. Previous studies have demonstrated that development of the agitated phenotype is influenced by strain and the time post-surgery^{27,28}. Future efforts should include a comprehensive battery of behavioral tests examining locomotion, activity, and anxiety, as well as circadian rhythm and hedonic behaviors, in response to OB and anti-depressant treatment, to further characterize the phenotype and validity of the model.

Although many studies have investigated the role of rTMS frequency on brain excitability and function, less focus has been given to the role of intensity, perhaps because the mechanisms activated by rTMS have always been assumed to involve traditional forms of synaptic plasticity such as long-term potentiation and long-term depression, which require action potential firing²⁹. However, recent work in humans and in animal models has shown that LI-rTMS and MI-rTMS (subthreshold) can have a notable effect on the brain^{5,22,30,31}. In our study, both MI-rTMS and HI-rTMS reduced FST hyperactivity in the OB model despite being sub- and at-threshold, respectively, and causing different neurobiologic outcomes and recruiting different mechanisms of action, as described. These results contrast with previous studies showing that rTMS induced hyperactivity in the open field in intact, normal rats³² although extensive differences in stimulation parameters, coil geometry, model, and species preclude a useful comparison between these two studies. Nonetheless, reports of rTMS modulating activity in preclinical studies suggest that it will be important to elucidate whether the improvement in FST following MI and HI-rTMS was due to rescue of the OB-induced psychomotor agitation, or a more general effect due to increased psychomotor inhibition.

Our experiments do not allow us to determine which intensity (LI, MI, or HI) provides better outcomes in our model because the behavioral response was similar for both intensities. However, we speculate that MI-rTMS may provide longer lasting anti-depressant effects than HI-rTMS due to the increase in neurogenesis observed only at medium intensity. The lack of effect on neurogenesis of HI-rTMS suggests a transient anti-depressant effect, and is consistent with the occurrence of relapse in human patients once treatment has ceased. It will be important to carry out longitudinal studies to determine when anti-depressant effects are first observed and how long they persist. It would also be interesting to test a two-

step treatment protocol: initial delivery of HI-rTMS might provide rapid benefits, which could then be followed with MI-rTMS to integrate these benefits into long-term structural changes through increases in neurogenesis.

Our work confirms previous suggestions that MI-rTMS upregulates BDNF following OB, in various limbic structures including the frontal cortex and hippocampus^{12,33}, but the involvement of the protein in the behavioral phenotype is still unknown, just as the relationship of BDNF to depression in humans is still under debate^{33–36}. The present data argue against the possibility that increased BDNF level could be a simple consequence of increased locomotor activity following OB^{37–39}, because MI-rTMS reduced hyperactivity while increasing BDNF concentration. Rather, BDNF upregulation may be a beneficial response to MI-rTMS with increased neuroplasticity¹². The increase in BDNF induced by MI-rTMS may have been sufficient to drive neurogenesis in the hippocampus: Likely, BDNF levels and neurogenesis are linked in our study because hippocampal BDNF levels have been causally linked with neurogenesis in mouse models of learning, enrichment, and anxiety^{39–42}. Interestingly, MI-rTMS increased the number of newly generated cells detected 3 weeks after the EdU injection but did not selectively increase the proportion of these cells that differentiated into neurons. Future work could examine whether MI-rTMS increases proliferation or simply increases the survival of newly born cells, because this result has implications for understanding the integration of newly born cells into the hippocampal network^{43,44}. It will also be interesting to investigate possible cognitive changes following OB and MI-rTMS treatment, because increased neurogenesis is associated with changes in learning and memory⁴⁵.

Our negative neurobiologic outcomes for HI-rTMS contrast with previous studies that showed increases in both BDNF^{46,47} and neurogenesis⁴⁸ following rTMS using a human coil. The different cellular responses to stimulation at similar intensities may be due to differences in the focality of the coils. We used a commercial rat coil (MagVenture) that not only delivers a broader field than our small animal coils (Fig. 1) but also differs in shape from the conventional human coils (such as figure-of-eight coils or large circular coils) used in previous studies⁴⁹. We also did not observe an increase in brain BDNF levels following LI-rTMS, consistent with previous work showing that very low-intensity stimulation may be brain region specific: LI-rTMS upregulates BDNF in the visual cortex, midbrain, and cerebellum but not in the retina^{50–52}, and the lack of upregulation of BDNF in the hippocampus is consistent with our previous study showing no change in hippocampal dendritic spines following LI-rTMS⁵³. Nonetheless, we cannot rule out a localized upregulation in the dentate gyrus with these intensities,

which was not detected in our dissection of the whole hippocampus. Overall, our findings highlight that the range of mechanisms called into play by different rTMS intensities has implications for outcomes in different brain regions that may receive different field strengths simply as a result of being at a distance from the focal point of stimulation⁵⁴.

Our serum analysis identified several metabolites that were altered in response to OB and in response to 1 or more rTMS frequencies. However, the concentrations of only 2 metabolites, AABA and 3-methylhistidine, were increased and decreased, respectively, by OB and restored to baseline levels by HI-rTMS treatment but not by LI-rTMS or MI-rTMS. This finding further highlights that MI-rTMS and HI-rTMS have specific effects on the different brain and peripheral markers of agitated depression modeled in our study, even though they may eventuate in similar behavioral outcomes. Interestingly, AABA has been identified as a potential biomarker for treatment-resistant depression in humans⁵⁵, consistent with the clinical population treated with rTMS^{56–58}. Therefore, evidence from animal and human studies suggests that AABA and 3-methylhistidine may serve as biomarkers in humans for phenotyping depression. Further longitudinal work could determine if AABA and 3-methylhistidine have utility for monitoring treatment response to rTMS or precision medicine approaches to brain stimulation interventions^{56–58}.

Our IPA identified glutamate degradation, glutamate-dependent acid resistance, GABA receptor signaling, and 4-aminobutyrate degradation as systems that were both downregulated following OB surgery and upregulated by HI-rTMS and, to a lesser extent, MI-rTMS. Consistent with our findings, changes in GABAergic and glutamatergic processes in the brain have been reported following OB^{59–62} and in the brain and blood of depressed patients⁶³. Furthermore, rTMS in rodents and in humans can selectively alter GABA and glutamate levels in the brain and blood^{64–66}. This result adds to clinical evidence that behaviors associated with depression may be mediated by GABAergic and glutamatergic systems^{66–70}.

The present findings and prior work suggest that significant changes occur in glutamate and other amino acids in the blood of depressed patients⁷⁰. However, the implications of this outcome are uncertain because peripheral measures do not necessarily reflect brain activity^{71,72}. A possible explanation is that activation of the hypothalamic-pituitary-adrenal axis and the autonomic nervous system impacts peripheral metabolite levels in the blood^{73–76}. For example, in rats, activation of the sympathetic nervous system increases the activity of glutamate pyruvate transaminase and glutamateoxaloacetate transaminase in the blood. These enzymes accelerate the conversion of glutamate to 2-ketoglutarate, resulting in a net decrease in plasma glutamate levels. Prior work

demonstrates that corticotrophin-releasing hormone and adrenaline reduce blood glutamate concentrations in rats⁷⁷. This explanation is compatible with our pathway analysis, implicating glutamate and GABA pathways in OB and in MI-rTMS and HI-rTMS-induced recovery.

In summary, our work suggests that the OB murine model may have utility as a model for agitative, treatment-resistant depression. Future studies with exhaustive behavioral testing could assist with the validation of this model. Variations in rTMS intensity appear to alter brain and metabolomic effects in the OB mouse model of agitated depression. Further work in this area could assist with the optimization of clinical rTMS delivery. Peripheral metabolomic markers might have utility as biomarkers for the diagnosis of specific forms of depression and in monitoring or modifying rTMS treatment. Further translational studies are warranted.

Disclaimer

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A.H., K.M., A.G., and J.R. performed the mouse rTMS experiments and behavioral analyses. A.H., K.M., A.G., and J.R. performed the 5-hydroxytryptamine, BDNF, and neurogenesis analyses. D.R.L., A.J.A., D.S.-C., and P.E.C. completed the metabolomic studies with enzyme-linked immunosorbent assay and ultrahigh pressure liquid chromatography-driven targeted metabolomics. D.R.L., A.J.A., D.S.-C., and P.E.C. completed and interpreted the IPA analysis. A.H., D.R.L., J.

R., and P.E.C. wrote the manuscript with contributions from K.M., A.G., A.J.A., and D.S.-C. J.R., D.S.-C., and P.E.C. designed the study and obtained the necessary funding. All authors commented on and approved the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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