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A little goes a long way: Neurobiological effects of low intensity rTMS and implications for mechanisms of rTMS

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ABSTRACT

Repetitive transcranial magnetic stimulation (rTMS) is a widespread technique in neuroscience and medicine, however its mechanisms are not well known. In this review, we consider intensity as a key therapeutic parameter of rTMS, and review the studies that have examined the biological effects of rTMS using magnetic fields that are orders of magnitude lower that those currently used in the clinic. We discuss how extensive characterisation of "low intensity" rTMS has set the stage for translation of new rTMS parameters from a mechanistic evidence base, with potential for innovative and effective therapeutic applications. Low-intensity rTMS demonstrates neurobiological effects across healthy and disease models, which include depression, injury and regeneration, abnormal circuit organisation, tinnitus etc. Various short and long-term changes to metabolism, neurotransmitter release, functional connectivity, genetic changes, cell survival and behaviour have been investigated and we summarise these key changes and the possible mechanisms behind them. Mechanisms at genetic, molecular, cellular and system levels have been identified with evidence that low-intensity rTMS and potentially rTMS in general acts through several key pathways to induce changes in the brain with modulation of internal calcium signalling identified as a major mechanism. We discuss the role that preclinical models can play to inform current clinical research as well as uncover new pathways for investigation.

1. Introduction

Repetitive Transcranial Magnetic Stimulation (rTMS) has grown as a field in neuroscience in recent years, with a wide range of tools and protocols available, some of which provide therapeutic effects in humans across a range of neurological and neuropsychiatric conditions (Lefaucheur et al., 2020). This has resulted in Food and Drug Administration (FDA)-approval for the use of rTMS to treat depression (O'Reardon et al., 2007), obsessive compulsive disorder (Carmi et al., 2018), and nicotine addiction (FDA et al., 2021). However, there is still much to be understood about the mechanisms behind these therapeutic effects, as unlike conventional drug discovery which follows a clearly laid out pathway through extensive safety and preclinical testing, the main body of rTMS research has been conducted in humans without *in vitro* and *in vivo* testing.

Repetitive TMS came out of single pulse TMS, which delivers high intensity single pulses to generate visible muscle twitches that can be used to measure conductance in motor pathways, measure motor evoked potentials, and map the motor areas of the brain, for example after a stroke (Barker et al., 1985; Hess et al., 1986). The use of high intensity magnetic fields was incorporated by default into rTMS, and by analogy with synaptic plasticity mechanisms that were beginning to take hold at the time (e.g. Bliss and Lømo, 1973; Barrionuevo et al., 1980). As a result, lower intensities were never seriously investigated despite a history of low intensity pulsed magnetic fields (PMF) being effective in a range of organs and in particular for bone healing and pain relief (e.g. Barker et al., 1984; Sharrard, 1990; Riva Sanseverino et al., 1992; Jorgensen et al., 1994) (Table 1).

However, in the past 10 years, there has been an increase in *in vivo* and *in vitro* work using rodents that has allowed greater understanding of the cellular and molecular effects of rTMS, and insight into potential for new applications. Early studies confirmed that long-term depression (LTD) and long-term potentiation (LTP)-like changes in excitatory and inhibitory neurons following rTMS in rodent brain slices (Thickbroom, 2007; Vlachos et al., 2012; Lenz et al., 2015, 2016). However an additional and perhaps undervalued benefit of the increasing number of preclinical studies has been the exploration of parameter space in a way that is not ethically or financially feasible in human participants.

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Table 1
Comparison of Pulsed Magnetic Field (PMF) and Low-Intensity Repetitive Transcranial Magnetic Stimulation (LI-rTMS) parameters.

	PMF	LI-rTMS
Common Nomenclature	Pulsed magnetic fields (PMF); Electromagnetic fields (EMF); extremely low frequency (ELF-) EMF	Low-intensity rTMS
Intensity	<15 mT	1–150 mT
Coil (Caveat: focality is not a factor for *in vitro* stimulation)	Hemholtz coil (2 coils above and below an area producing uniform magnetic field) MRI magnets Non-focal stimulation (e.g. whole rodent body, whole head etc.)	Unidirectional coil Stimulation above a targeted (focal) stimulation area
Common Frequencies	40–75 Hz, 50 Hz commonly (mimics electronic device frequencies)	Low-frequency (1–5 Hz); high frequency (>10 Hz) and patterned protocols (e.g. theta burst stimulation, intermittent or continuous (iTBS, cTBS) and biomimetic high frequency stimulation (BHFS)) (Defined based on influence on cell excitability)
Stimulation Time	Wide range: 15mins–24 h; 1 h, commonly	Commonly 3–10 min per session
Historical Research Context	1. Concern over potential negative effects of background electromagnetic radiation emitted from electronic devices or sources (often <1 mT intensities, long exposure times, stimulation of developing embryos/ assessment of cell death) 2. Therapeutic PMF devices for pain relief and accelerated healing in the body (i.e. peripheral nervous system, muscle, bone)	Neurobiological therapeutic context – e.g. treatment of depression, neurobiological disorders. Generally targeted toward shorter treatment times and, in the case of patterned frequencies, stimulation that mimics endogenous brain signals

Research in this space has identified intensity parameters that engage specific brain mechanisms that were not anticipated from human studies. These mechanisms have led to new insights into how the brain is affected by electromagnetic induction, and inform translation.

In this review, we consider intensity as a key therapeutic parameter of rTMS, and review the studies that have examined the biological effects of rTMS using magnetic fields that are orders of magnitude lower that those currently used in the clinic (termed high-intensity (HI-) rTMS). We discuss how extensive characterisation of "low intensity" rTMS has set the stage for translation of new rTMS parameters from a mechanistic evidence base, with potential for innovative and effective therapeutic applications.

1.1. Low intensity magnetic fields

Due to the nature of electromagnetism, definitions relating to intensity are difficult to characterise. Intensity of magnetic stimulation can be measured in different ways, namely the strength of the magnetic field (in Tesla or Gauss), the rate of change of the coil current or the coil magnetic field (eg. Volts/second or Tesla/second), and these two factors together determine the induced electrical field (Volts per metre). Each factor is useful in different contexts.

Measuring the magnetic field is relatively straight forward and can be done with basic portable equipment (a gaussmeter), therefore the magnetic field is probably the most practical in a clinical context. The electric field is arguably the most biologically relevant measure of rTMS intensity because the intensity of the induced electric field determines whether an action potential is triggered (although see section 4 about magnetic field effects). However, the induced electric field is difficult to measure directly and generally involves complex computational modelling. Nonetheless, models evaluating electric field strength have been highly informative in understanding electromagnetic field interactions with brain anatomy and tissue properties. The rate of current change (dI/dt) or magnetic field change (dB/dt) is a key determinant of electromagnetic field strength because the rate of change determines the intensity of the induced field. For example, a strong magnetic field with a slow rate of change will induce a low intensity electric field. Conversely, a weak magnetic field with a fast rate of change will induce a strong electric field. Rate of change values can be used to estimate the induced electric field strength but is more useful in the context of engineering and coil design, because the coil design properties may limit the rate of change (e.g. fast rates of change produce heat, which can damage the coil or the experimental subject).

This review focusses on "low-intensity (LI-)" rTMS which we define "biologically" as magnetic stimulation delivered at intensities that do not directly trigger an action potential. In quantitative terms, LI-rTMS includes intensities that result in an induced electric field less than 28 V/m, a limit based on computer models which suggest that 28 V/m is the minimum intensity required to induce an action potential in neurons (Radman et al., 2009). In section 2 we briefly discuss a subsection of PMF literature, another form of subthreshold magnetic stimulation which has some overlap and parallels with LI-rTMS although Table 1 outlines the differences between techniques. Additionally, it is important to differentiate between concepts of "subthreshold for action potentials", which is the definition we use here, and "subthreshold for motor evoked potentials", which is a concept used in conventional rTMS in human subjects. Although conventional rTMS is often applied at 70–100% of motor threshold, the intensity of the induced electric field is still above the limit of 28 V/m and therefore induces action potentials in underlying neurons, even though there is no resulting muscle contraction. Sub-motor threshold stimulation is of interest in its own right, and may share mechanisms with rTMS and LI-rTMS, but is beyond the scope of the present review.

The coils that deliver LI-rTMS include the custom rodent coils characterised by Tang et al. (2016b) and the majority of experiments with custom miniaturised coils in animal and cell models. These coils deliver magnetic fields between 1 and 150 mT, which is one or two orders of magnitude lower than 'conventional' human rTMS (HI-rTMS). We note that novel coil designs continue to be developed and are beyond the scope of this review (e.g. Khokhar et al., 2021), although to our knowledge there is not yet a focal miniaturised coil that can induce rTMS at suprathreshold levels for an extended period of time. It is also currently not known what the minimum biologically meaningful electric field may be.

A further consideration regarding stimulation intensity is that the induced magnetic and electric fields decrease as a function of distance from the coil: high intensity rTMS generates electric fields that induce action potentials in the neurons at the stimulation site, while also delivering subthreshold intensities in surrounding tissue. Electric field modelling of HI-rTMS coils often lacks detail regarding lower level field strengths, therefore it is unclear how much of the brain receives stimulation in the range of LI-rTMS during HI-rTMS protocols. However interpolation across several models of HI-rTMS in humans suggest stimulation at LI-rTMS levels likely occurs widely across the brain: stimulation of a human head model with a Figure-8 coil (50% of maximum stimulator output) (Smith and Peterchev, 2018) induces electric fields equivalent to those of an 8 mm, 18 mT LI-rTMS coil (Madore et al., 2021; Moretti et al., 2021) in areas at least 5–6 cm away

from the targeted area. Therefore, LI-rTMS research may not only make important therapeutic contributions in its own right, but may also shed light on mechanisms of HI-TMS that have been obscured by the focus on action potential induction.

In addition, electric field measurements of some sham coils also show biologically relevant induced electric fields in the brain (Smith and Peterchev, 2018). In one case, modelling of HI-rTMS 'sham' conditions where spacers are used to position the coil 3.3 cm above the scalp indicated that the induced electric fields remain strong enough that they may be able to induce action potentials themselves (i.e. >28 V/m, Radman et al., 2009) (Boucher et al., 2021). Therefore induction of subthreshold electric fields with 'active' sham conditions is also worth considering and are discussed further in section 5.

Even though LI-rTMS does not induce action potentials, there is significant evidence that LI-rTMS is able to induce behavioural changes in animal models, as well as a range of cellular, molecular and genetic changes *in vitro* and *in vivo*. In this review we discuss the literature that has used low intensity magnetic fields first in the form of PMF, then as LI-rTMS, which differ primarily their protocols and focality (Table 1). Approaches range from *in vitro* single-cell recordings to *in vivo* models of disorders such as brain injury and depression. We provide an inclusive review of the possible mechanisms of low intensity magnetic field stimulation, including both electric and magnetic field-specific effects.

2. Pulsed magnetic fields

PMF pioneered the study of electromagnetic field interactions with biological tissue, mostly using Helmholtz coils to deliver non focal low intensity stimulation to a range of cells and tissues. Below, we briefly review studies of PMF in CNS tissue *in vivo* and *in vitro*, restricting our focus to studies using intensities higher than 1 mT in order to maximise relevance to LI-rTMS.

2.1. Excitability

PMF appears to be able to influence neuronal excitability following a single stimulation session (Wieraszko, 2004 (0.03–0.5 Hz, 9–15 mT); Ahmed and Wieraszko, 2008 (0.16 Hz, 15 mT)), which may be tied to cell signalling changes such as increased glutamate turnover (Wieraszko et al., 2005 (0.03–0.5 Hz, 9–15 mT)) and increased cAMP concentration (Hogan and Wieraszko, 2004 (0.16 Hz, 15 mT)). However, it is unlikely that excitability changes are due to the modulation of membrane voltage-gated channels as current recordings and channel activation were not significantly altered following PMF in cultured entorhinal cortex neurons (Luo et al., 2014 (50 Hz, 1 mT and 3 mT)). Rather, changes to calcium dynamics driven by intracellular calcium release appear to be an underlying mechanism (Morabito et al., 2010 (50 Hz, 1 mT); Luo et al., 2014). Internal calcium concentration changes were also observed in an astrocytic cell line following PMF, demonstrating changes in non-neuronal signalling (Aldinucci et al., 2000 (50 Hz, 3 mT)).

Increased excitability following PMF has also been shown at network levels and behaviourally. In humans, an increase in intracortical facilitation was shown after PMF, but not sham stimulation (Capone et al., 2009 (75 Hz, 1.8 mT)) and functional magnetic resonance imaging (fMRI) showed changes in task-induced functional brain activation, in areas associated with task-specific performance following PMF, although task performance itself did not differ between stimulation groups (Legros et al., 2015 (60 Hz, 1.8 mT and 3 mT)). Following PMF in rats, spatial memory was improved which suggests that increased excitability may facilitate memory acquisition (Liu et al., 2008 (50 Hz, 2 mT)). However PMF stimulation is not consistently effective or beneficial. There is some evidence that PMF has no effect on the brain: visual evoked potentials were unaffected by PMF in healthy humans suggesting that stimulation did not alter visual cortical excitability Glover et al. (2007) (490 Hz for 0.5s repeated every 5s, 2.8 mT). The tendency for positive publication biases, especially in neuromodulation literature also suggests that several other instances of lack of effects likely occur. There have also been reports of impaired spatial memory (Lai, 1996 (60 Hz, 0.75 mT); Jadidi et al., 2007 (50 Hz, 8 mT); Fu et al., 2008 (50 Hz, 1.1–2 mT)), memory consolidation (Foroozandeh et al., 2013 (50 Hz, 8 mT)) and place learning (Lai et al., 1998 (60 Hz, 1 mT)) with PMF compared to sham stimulation in animal models.

2.2. Cell survival and proliferation

Following injury and stroke models, PMF stimulation has evidenced neuroprotective properties and reduced inflammatory responses Grant et al. (1994) (75 Hz, 2.8 mT); Mert et al. (2006) (trains of increasing frequency: 1 Hz, 10 Hz, 40 Hz and 100 Hz, 1.5 mT); Rasouli et al. (2012) (3 ms bursts of 27.12 MHz twice a second, 40 ± 6 V/m); Pena-Philippides et al. (2014) (2 ms bursts of 27.12 MHz twice a second, 3 ± 0.6 V/m); Cichoń et al. (2017) (40 Hz, 7 mT); Gessi et al. (2019) (75 Hz, 1.5 mT). For example increases in nitric oxide following PMF Cho et al. (2012) (60 Hz, 2 mT); Bragin et al. (2015) (3 ms 27.12-MHz bursts repeating at 5hz, 6 ± 1 V/m) mediate increased blood flow and tissue oxygenation (Bragin et al., 2015) which could improve recovery following ischemic stroke. Similarly, increased adenosine receptor expression Varani et al. (2012) (75 Hz, 1.5 mT and 3 mT), increased neurite outgrowth Greenebaum et al. (1996) (bursts of asymmetric, 220 μs-wide pulses repeated at 15 Hz and 25 Hz, 4 mT); Macias et al. (2000) (bursts of asymmetric, 220 μs-wide pulses repeated at 25 Hz, 1.67 V/m), altered catalase and cell differentiation activity in undifferentiated cells (Morabito et al., 2010), as well as proliferation and neurotrophic gene expression changes Liu et al. (2015) (50 Hz, 2 mT) have been found across several tissue types and stimulation parameters. These changes could be beneficial in a post-injury or regeneration scenarios. However, chronic stimulation, but not single session stimulation is also associated with decreased cell viability and increased reactive oxygen species (ROS) generation compared to sham Zeng et al. (2017) (50 Hz, 2 mT), emphasising timing and dose dependent changes.

2.3. Metabolite and neurotransmitter modulation

Pharmacological studies in animals have suggested that PMF may also change how neurotransmitters are metabolised or how receptors are expressed, resulting in behavioural changes. Pešić et al. (2004 (50 Hz, 6 mT)) showed rats had altered stereotypic responses to moderate doses of amphetamine after stimulation which may be due to changes in neurotransmitter uptake or altered enzymatic cascades determined by Ca^{2+}- dependent processes. Changes to neurotransmitter uptake have been directly demonstrated following PMF: stimulation of rats resulted in reduced cholinergic uptake in the frontal cortex and hippocampus (Lai et al., 1993 (60 Hz, ≥0.75 mT); Lai and Carino, 1999 (60 Hz, 0.5, 1.0, 1.5, or 2.0 mT)). The change in cholinergic uptake was present only at 2 mT intensity following 60 min exposure, but when exposure time was increased, lower intensity stimulation also demonstrated reduced uptake, showing that intensity and duration of exposure interact, with a possible dose threshold (intensity x number of pulses) (Lai and Carino, 1999). Similarly, whole body stimulation at 10 Hz was reported to significantly increase dopamine and serotonin turnover compared to sham in the rat frontal cortex, although not the striatum (Sieroń et al., 2004 (1.8–3.8 mT)). The authors suggested this was due to increased neurotransmitter synthesis in the frontal cortex (Sieroń et al., 2004), but may also reflect changes to neurotransmitter metabolism. PMF stimulation of rats also potentiated morphine-induced conditioned place preference which may be reflective of increased opioid receptors (Lei et al., 2005 (20 Hz, 1.8 mT and 50 Hz, 2.2 mT)). Changes to opioid receptor expression could augment the rewarding effects of morphine to prolong conditioned place preference after withdrawal (Lei et al., 2005). There is still limited evidence to fully characterise mechanisms behind drug-related changes, but PMF stimulation does appear to modulate

neurotransmitter activity.

Overall, the PMF literature is quite broad, but there are several studies which show significant biological changes induced in the brain by non-focal, low-level electromagnetic stimulation at various frequencies. Common mechanisms such as internal Ca^{2+} signalling, modulation of neurotransmitter metabolism and receptor expression, and upregulation of anti-inflammatory, pro-survival mechanisms have been found across several studies. These changes also parallel some seen in the LI-rTMS literature, suggesting significant overlap between the two techniques (Table 1).

3. LI-rTMS

An advantage of LI-rTMS over PMF is that it can deliver focal stimulation to limited areas of the brain. In rodent models this involves using simple circular coils that are small enough to fit on the head of a mouse or rat to deliver focal stimulation (Tang et al., 2016b; Poh et al., 2018; Madore et al., 2021). For example, electric field models of an 8 mm LI-rTMS coil delivering 18 mT to a mouse show that induced electric fields are restricted mostly to cortical tissue, with penetrance of 2.5 mm into the cortex before electric field drops below 0.2 V/m (Moretti et al., 2021). At 4 mm below the base of the coil, the magnetic field is measured at 2 mT and induced magnetic fields greater than 1 mT remain limited to underneath the coil (Poh et al., 2018). Coils delivering LI-rTMS at higher intensities will likely have a greater spatial distribution of the electromagnetic field than the 18 mT coils described above, but still remain more focal than PMF stimulation. A summary table of LI-rTMS studies can be found in Table 2. Most LI-rTMS protocols are the same as those used with HI-rTMS, with the exception of biomimetic high frequency stimulation (BHFS), a patterned protocol with trains of pulses delivered at 6–10 Hz (Martiny et al., 2010) that appears excitatory and was initially designed to mimic endogenous patterns of electrical fields around activated nerves during exercise.

3.1. Excitability

3.1.1. Excitatory LI-rTMS increases evoked potentials

Unlike HI-rTMS, LI-rTMS is subthreshold, which we define here as meaning that it does not induce action potentials. Despite this, there is still evidence that LI-rTMS alters neural excitability. In HI-rTMS studies, modulation of neural excitability is most often assessed through the effect on evoked potentials, particularly motor evoked potentials (MEPs). Despite not inducing action potentials, excitatory LI-rTMS has been shown to alter corticospinal excitability in rodents through the increase of motor evoked potentials (MEP) amplitudes (normalised to baseline) (Tang et al., 2016b) as well as changes to visual pathway excitability through changes to late visual evoked potentials (VEPs) (Makowiecki et al., 2018). In a guinea pig model of tinnitus, electrophysiological recordings showed that spontaneous firing in the medial geniculate nucleus (MGN) was significantly increased after multiple sessions of both 1 Hz and 10 Hz rTMS over the prefrontal cortex (Mulders et al., 2019). Furthermore, c-Fos expression, a marker of neuronal activity is upregulated after LI-rTMS (Grehl et al., 2015; Dufor et al., 2019; Moretti et al., 2021). Therefore, even at low intensities and without directly evoking action potentials, LI- rTMS (like PMF) is able to induce changes to neural excitability in the brain.

Interestingly, excitability changes following LI-rTMS are affected by brain state during stimulation, a concept which has been highlighted in the broader neuromodulation literature (e.g. Prichard et al., 2014; Gill et al., 2015; Friehs and Frings, 2019). LI-rTMS modulation of visual pathway excitability was dependent on the visual activity of the mouse (Makowiecki et al., 2018). Specifically, changes to VEPs following LI-rTMS occurred only when the mice received concurrent visual input (i.e. not in the dark) (Makowiecki et al., 2018). LI-rTMS effects may be enhanced by controlling brain state at the time of stimulation, and this raises the possibility of identifying biomarkers that may predict response to stimulation. These applications are also of current interest in HI-rTMS research (e.g. Arns et al., 2012; Bergmann et al., 2016; Thut et al., 2017; Bergmann, 2018; Garnaat et al., 2019), with greater consideration toward combining stimulation with electrophysiology or neuroimaging measures such as EEG to either predict the likelihood of a beneficial response, or tailor timing of stimulation to optimise the outcome.

In order to understand what may underly LI-rTMS induced changes to excitability, several studies have investigated cellular changes following stimulation at several timepoints in order to understand acute and chronic changes.

3.1.2. LI-rTMS alters membrane properties

Electrophysiological recordings (whole-cell patch clamp electrophysiology) taken immediately following 10 Hz LI-rTMS confirm that LI-rTMS does not induce action potentials (Tang et al., 2016a). The recordings also demonstrate that the resting membrane potential itself does not change following a session of LI-rTMS. Rather LI-rTMS hyperpolarises the action potential threshold and less change in membrane potential is required to trigger an action potential. Factors that modulate lowered action potential threshold include activation of voltage-gated sodium channels or voltage-gated calcium channels. The fact that LI-rTMS induced lowering of the action potential threshold immediately after stimulation in Tang et al. (2016a) suggests that LI-rTMS may act on the voltage-sensing mechanism of the voltage-gated sodium channels or voltage-gated calcium channels to have them activate at more hyperpolarised voltages. The rate of evoked spike-firing frequency also increased following LI-rTMS, although LI-rTMS did not induce a faster afterhyperpolarisation (Tang et al., 2016a). Authors suggest that modulation of A-type potassium channels (K_A) could play a role in controlling the increase in evoked-spike firing frequency, but further studies would be needed to elucidate (Tang et al., 2016a).

The fact that resting membrane potential does not increase may seem surprising, however the focus on somatic activity overlooks processes occurring outside of the cell body that can modulate excitability. Dendritic and axonal processes also regulate membrane excitability. Studies using transcranial direct current stimulation, which also delivers subthreshold stimulation, show alterations to dendritic potentials that influence plasticity (Bikson et al., 2004; Kronberg et al., 2017) and raise the possibility that changes to the axonal initial segment may contribute to changes in membrane excitability (Hoy and Fitzgerald, 2015; Bikson et al., 2019). Therefore electrophysiological changes outside of the soma (i.e. dendritic and axonal excitability) may also contribute to rTMS-induced changes in cell firing probability.

Another factor to consider in LI-rTMS is that stimulation is generally given chronically in daily sessions over several days to weeks. However, research investigating membrane properties is currently limited to single session (acute) LI-rMS, so it is still unclear what the long-term effects of chronic LI-rTMS may be. Given that homeostatic mechanisms may be called into play after a stimulation session, membrane changes may depend on complex interactions between the number of stimulation sessions and time post stimulation.

3.1.3. LI-rTMS modulates internal calcium release

3.1.3.1. Direct measures. Changes to calcium signalling have been shown immediately following LI-rTMS in both neurons (Grehl et al., 2015; Ye et al., 2020) and astrocytes (Clarke et al., 2017b) *in vitro*. A single 10 min stimulation of cortical neurons *in vitro* with 10 Hz, BHFS and cTBS induced immediate and significant increase in intracellular Ca^{2+} concentration (Grehl et al., 2015), assessed by live-cell imaging using a ratiometric intracellular calcium indicator. In a separate study, a single 30 min stimulation of continuous 10 Hz LI-rTMS significantly increased intracellular Ca^{2+} concentration for at least 3 h (Ye et al., 2020), as measured by colorimetric assays performed on collected media. In contrast, when 10 Hz was delivered intermittently during the

Table 2
Summary of LI-rTMS studies and their main findings.

Study - N	Subject - **Stimulation Site**	Condition	Session No.	rTMS Parameters	Intensity (Intensity @ Target)	Measurements	Main Findings
Bates et al. (2012) $N = 22$ (6 per stim group, 4 controls)	Rat, Male Spontaneously Hypertensive Rat - Ipsilateral or Contralateral injury site	Ischemic Stroke	8	20 Hz combined with BHFS, 1 Hz or Sham - 10 min daily	8 mT (6 mT)	Functional deficit behavioural tests, IHC: IBA1, ED1, GFAP, TUNEL	↑ macrophage infiltration (↑ ED1) after 20/1 Hz and 20 Hz/BHFS
Clarke et al. (2017a) 17–34 cells per condition (3–4 culture replicates)	Mouse, In vitro cell culture, primary astrocyte - Astrocytes	Healthy and Scratch Assay	14	1 Hz, cTBS, 10 Hz, BHFS, Sham - 10 min daily	18 mT	Calcium imaging, scratch test, BrdU, cell culture composition, cell morphology	1 Hz: ↑ internal Ca^{2+} concentration. Released from intracellular stores. Transient (24hr) reduction in astrocytic swelling after scratch assay
Clarke et al. (2017b) $N = 80$ (4–5 per condition)	Mouse, C57BL6/J mice - Ipsilateral injury site	Cortical Stab Injury	14	1 Hz, Contralateral 1 Hz, BHFS, Sham - 10mins daily	12 mT (5.4 mT, 1 Hz; 4 mT, BHFS)	IHC: GFAP, IBA1, CS56	Changes in astrocytic and microglia reactivity after cortical stab, but with sex and age differences.
Clarke et al., (2021) $n = 31$ over 6 culture runs	Mouse, In vitro, primary cortical cultures, astrocyte enriched - Astrocytes	Healthy	1	1 Hz - 10mins, 10 Hz - 1 min, 10mins, Sham	18 mT	Gene Expression Analysis, Protein expression immunofluorescence	21 of 125 genes downregulated after LI-rTMS. Genes related to inflammation, signalling, plasticity and cytoskeleton. Gene changes were reflected in protein levels - looked at Stim1, Orai3, Kcnmb4, and Ncam1. May be due to LI-rTMS-induced calcium level changes.
Cullen et al. (2019) Counts across 3–5 mice per treatment group	Mouse, Pdgfrα-CreERT2:: Rosa26-YFP - Midline	Healthy	14 or 28	10 Hz (60s), iTBS (192s), cTBS (40s), Sham - daily (600pulses)	120 mT	IHC: PDGFRα, EdU, TUNEL, OLIG2	14 days iTBS ↑ no. Newborn or newly differentiated OLs in M1, V2 and spinal cord in layers I, V and VI. Gradually drops to sham when stimulation stops. Maintained stimulation does not increase effect but increases maturation of OLs by improving survivability.
Cullen et al. (2021) Counts across 3–4 mice per group	Mouse, Pdgfrα-CreERTM:: Rosa26-YFP:: Myrffl/fl (Myrfl/fl) transgenic mice - Midline	Healthy	7–28	iTBS, Sham - 192s	120 mT	OL morphology, Block new OL addition. Computational simulation of conduction velocity, *ex vivo* CAP recordings	14 d & 28 d iTBS, but not 7 d: ↓ node length and ↑ periaxonal space in M1 and CC. *pms* the effect is maintained in CC but not M1. Effect is independent of new OL addition. iTBS ↓ conduction velocity, ↑ CAP amplitude of myelinated axons. ↑ synchronous AP arrivals. In contrast: Activity-dependent plastcity ↑ node length,↓ periaxonal space → ↑ conduction velocity.
Dufor et al., (2019) $n = 5–11$ per group	Mouse, In vivo cerebellum and *ex vivo* cerebellar explants	Olivocerebellar lesion	14	BHFS, Sham - 10 min daily	(In vivo: 8–10 mT, *ex vivo*: 10 mT)	IHC: V-GLUT2, c-Fos. Gene Expression	In vivo: BHFS → olivocerebellar reinnervation. Ex vivo: iTBS & BHFS → reinnervation.10 Hz: proximal reinnervation only. Reinnervated target cells show ↑ c-Fos & ↑ expression of genes related to axoneogenesis and post-lesion reinnervation after iTBS, BHFS. Cryptochrome DKO mice: no reinnervation after BHFS, need

(*continued on next page*)

Table 2 (*continued*)

Study - N	Subject - **Stimulation Site**	Condition	Session No.	rTMS Parameters	Intensity (Intensity @ Target)	Measurements	Main Findings
							cryptochrome for LI-rMS reinnervation.
Grehl et al. (2015) 12-18 images per group from 2 to 3 litters	Mouse, In vitro cell primary cortical neuron culture	Healthy	4	1 Hz, 10 Hz, 100 Hz, cTBS, BHFS, Sham - 10mins daily	(13 mT)	IHC: Caspase-3, TUNEL, GFAP, βIII Tubulin, Calbindin, SMI-32. Calcium Imaging, Gene Expression Array	LI-rTMS affects cell survival. 10 Hz and 100 Hz: ↑ apoptotic cells. 1 Hz: ↓ excitatory neuron survival, but no ↑ in apoptosis, ↓ neurite branching and outgrowth. 10 Hz, cTBS, BHFS: ↑ Ca^{2+} concentration, release of intracellular stores. 16 genes with altered expression changes, 15 associated with cell survival and apoptosis, or cell morphology and migration. 10 Hz: upregulated pro-apoptotic genes, downregulated anti-apoptotic genes. 1 Hz and BHFS: pro-survival changes to gene expression.
Grehl et al. (2016) 16 co-cultured explants (32 embryos across 3 litters)	Mouse, Ex vivo cerebellar explants	Olivocerebellar lesion	14	1 Hz, 10 Hz, Sham - 10 min daily	10 mT	IHC: c-Fos, Gene Expression changes	BHFS, 10 Hz: ↑ c-Fos. BHFS: ↑BDNF expression. 1 Hz: ↓Pax3, Sia2. 10 Hz: ↑ Sia2 and Sia4.
Heath et al., (2018) *n* = 9–16 per group	Mouse, C57Bl/6 J - Frontal Ctx	Depression	20	10 Hz, Sham - 3 min each weekday	LI: 12 (4 mT) MI: 90 (50 mT) HI:1.2 T (1 T)	Forced swim test; BDNF, plasma metabolites, IPA analysis	Improvement in depression-related behaviour: 50 mT and HI improved forced swim test, ↓ psychomotor agitation. Metabolites: 50 mT: ↑ hippocampal and frontal ctx BDNF and hippocampal neurogenesis. All intensities: altered plasma metabolite profile, IPA analysis shows altered metabolic pathways. Glutamine processing and glutamate signalling pathway changes across all intensities
Hong et al., (2018) *n* = 4–6 per group	Rat, In Vitro B50 neuroblastoma cells	B50 neuroblastoma	1	1 Hz, 10 Hz - 10mins	10 mT (2.2–2.4 mT)	Metabolite Assay	Changes in 18 metabolites detected. 1 Hz: ↓ 7 amino acids, and 5 other metabolites including cholesterol. 10 Hz: ↓ 5 amino acids that 1 Hz also affected, and ↓ 4 other metabolites.
Makowiecki et al., (2014) *n* = 22 per group	Mouse, C57Bl/6 J, EphrinA2A5−/−- Visual Ctx	Axonal Disorganisation	14	BHFS, Sham - 10 min daily	12 mT (8 mT)	Anatomical Tracing of cortical projections (V1 to SC), corticotectal and geniculocortical topography, ELISA (BDNF)	LI-rTMS improves the most disordered corticotectal projections in ephrin-A2A5$^{-/-}$ mice. ↓ Abnormally high dispersion of retrogradely labelled dLGN neurons. Selective for inappropriate efferents as no change in WT topography. ↑ BDNF in SC and Visual ctx in both ephrin and WT.
Makowiecki et al. (2018) *N* = 95 (48 WT; 47 EphrinA2A5−/−)	Mouse, C57Bl/6 J, EphrinA2A5−/−- Visual Ctx	Axonal Disorganisation	1	10 Hz, Sham - 10mins	(10.6 mT)	VEPs, IHC: parvalbumin	VEP changes with LI-rTMS only for WT with visual input - ↓ late response positive peak amplitude and ↑ in negative-deflecting peak.

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Table 2 (*continued*)

Study - N	Subject - **Stimulation Site**	Condition	Session No.	rTMS Parameters	Intensity (Intensity @ Target)	Measurements	Main Findings
							All genotypes: LI-rTMS ↑ parvalbumin positive densities in superficial cortical layers only when visual input.
Morellini et al. (2015) N not reported	Mouse, In vivo cerebellum and *ex vivo* cerebellar explants - Cerebellum	Healthy, olivocerebellar explant lesions	14 or 28	BHFS, Sham - 10 min daily	10 mT	Gait - catwalk; spatial learning; Morris water maze; Purkinje patch clamp; dendritic arbour dimensions	Healthy *in vivo*: LI-rTMS improved spatial memory in probe test. Purkinje cell dendritic arbours taller and greater surface area with LI-rTMS. Increased spine density w/LI-rTMS. Lesioned explants *in vitro*: LI-rTMS and BDNF treatment both induced climbing fibre reinnervation. LI-rTMS ↑ BDNF. rTMS + BDNF combination treatment - no additive effect.
Moretti et al. (2021) $N = 24$; $n = 6$ per group	Mouse, C57Bl/6 J, EphrinA2A5−/−- Visual Ctx	Axonal Disorganisation	14	BHFS, Sham - 10 min daily	18 mT	Progressive ratio task, IHC: c-Fos, DARPP32, TH. c-Fos density correlations	Ephrin-A2A5$^{-/-}$ mice ↑ last ratios vs. wildtype mice only in the second progressive ratio schedule (PR7). Ephrin-A2A5$^{-/-}$ mice ↓ c-Fos expression in the PrL, NAc core and shell on final day. C-Fos density correlated with task performance in sham group, but not rTMS.
Mulders et al. (2016) $N = 19$, 12 developed tinnitus ($n = 6$ per group)	Guinea Pig, Pigmented Guinea Pig - Contralateral Auditory Ctx	Tinnitus	10	1 Hz, Sham - 10 min daily (weekdays only)	90 mT	Single neuron recordings of spontaneous activity, BDNF, CAP audiograms, PPI, GPIAS test.	BDNF: No change. Hearing loss: No change. Behavioural signs of tinnitus reversed with LI-rTMS.
Mulders et al. (2019) $N = 24$, 18 developed tinnitus ($n = 5$–7 per group)	Guinea Pig, Pigmented Guinea Pig - Contralateral Prefrontal Ctx	Tinnitus	10	1 Hz, 10 Hz, Sham - 10 min daily (weekdays only)	90 mT	spontaneous recordings from MGN, parvalbumin and calbindin in PFC, CAP thresholds, GPIAS test	1 Hz and 10 Hz ↑ MGN firing. Dorsal PFC region of interest showed ↑ parvalbumin density in 10 Hz vs. 1 Hz. -ve correlation between calbindin density and MGN spontaneous firing rates.
Poh et al. (2018) ***N*** = 46 (12 WT; 32 EphrinA2A5$^{-/-}$)	Mouse, C57Bl/6 J, EphrinA2A5−/−- Visual Ctx	Axonal Disorganisation	14	BHFS, Sham - 10 min daily	12 mT	Visual Learning Task (Y Maze), Locomotor activity, Anatomical Tracing of projections, corticotectal and geniculocortical topography, Visuomotor head-tracking	LI-rTMS: only improved disordered projections when there was no task. LI-rTMS ↓ dispersion in dLGN and improved head-tracking w/o task effect. Ephrin-A2A5$^{-/-}$ mice had lower accuracy and # trials performed. LI-rTMS ↑ cumulative number of tasks performed in visual learning task for ephrin mice closer to wildtype levels.
Poh et al., (2019) n = 5–6 per group	Mouse, C57Bl/6 J - Visual Ctx	Healthy	1	10 Hz, Sham - 10 min + HI-rTMS (6 min, 10 Hz)	12 mT	LC-MS/MS	LI-rTMS: No sig changes. HI-rTMS: ↓ 5-HT turnover in the cortex, and altered DOPAC concentrations in the hippocampus and striatum. ↓ α-aminoadipic acid concentrations in the hippocampus and a ↓ serine, threonine, sarcosine, aspartate and Glu concentrations in the striatum
Rodger et al., (2012) n = 4–16 per group	Mouse, C57Bl/6 J, EphrinA2A5−/−- Superior Colliculus	Axonal Disorganisation	14,1	BHFS, Sham - 10 min daily	10 mT (6 mT)	Visual tracking, Anatomical Tracing, Electrophysiological	Chronic LI-rTMS: restored normal visual tracking behaviour in ephrin-

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Table 2 (*continued*)

Study - N	Subject - **Stimulation Site**	Condition	Session No.	rTMS Parameters	Intensity (Intensity @ Target)	Measurements	Main Findings
						recording, ELISA (BDNF, nNOS, GABA)	A2A5$^{-/-}$ mice, ↓ ectopic retinocollicular terminals in ephrin mice, altered functional response in ephrin-A2A5$^{-/-}$ mice but not WT. ELISA: ↑BDNF, ↑ nNOS in retina and SC, changes to GABA SC
Seewoo et al. (2018) *N* = 6, longitudinal design	Rat, Male Sprague Dawley Rats - Right hemisphere	Healthy	1	1 Hz, 10 Hz, cTBS or BHFS, Sham - 10mins	(13 mT)	rs-fMRI	LI-rTMS protocols changed the DMN, pattern of change was frequency dependent and similar to HI-rTMS DMN changes.
Seewoo et al., (2019) *n* = 9 per group	Rat, Male Sprague Dawley Rats - Right hemisphere	Healthy	15	1 Hz, 10 Hz- 10mins daily (weekdays only)	(13 mT)	rs-fMRI, MRS	10 Hz: ↑ Gln after 7 days stim, back to baseline after 14 d stim. 1 Hz: ↓ Gln, Glu and Glx at seven days *pms*. 10 Hz: ↑ RSN connectivity across 3 networks, back to baseline 7 d *pms*. 1 Hz: ↓RSN connectivity across 3 networks, sustained 14 days *pms*.
Seewoo et al. (2021) *N* = 88 Depressed: *n* = 19–22 per group; Healthy: 8–9 per group	Rat, Male Sprague Dawley Rats - Right hemisphere	Healthy, Depression (CRS model)	15	1 Hz, 10 Hz, Sham - 10mins daily (weekdays only) 10 Hz–10mins, 3 x daily (weekdays only)	(13 mT)	dMRI, IHC: myelin basic protein	Healthy: 10 Hz and 1 Hz alter white matter tracts: ↑ fractional anisotrophy 1 or 2 weeks *pm*. Altered diffusion and kurtosis parameters. 10 Hz > 1 Hz effects. Depression model: white matter maturation-related changes delayed. Accelerated 10 Hz stimulation rescues white matter delays, ↑ myelin basic protein levels
Sykes et al. (2013) *N* = 20 (10 per genotype)	Mouse, C57Bl/6 J, EphrinA2−/−- Hippocampus	Axonal Disorganisation	35	BHFS, Sham - 10 min daily	10 mT (6 mT)	Visual discrimination task - Y maze task; Golgi staining	No change in learning or reversal learning in Y-maze visual task between treatment or genotype. No change in dendritic spine density with rTMS, or between genotype.
Tang et al. (2015) *N* = 34, *n* = 5–8 per group	Mouse, C57Bl/6 - Operated Eye	Optic Nerve Crush	14 or 7	BHFS, Sham - 10 min daily	12 mT (7.4–1.8 mT)	BDNF, βIII tubulin, RGC survival, axonal growth	No change in RGC survival, no axon regeneration, no change in BDNF in retina or optic nerve
Tang et al. (2016a) *N* = 4	Rat, Male Sprague-Dawley - Left Motor Ctx	Healthy	1	10 Hz - 3 min (1800 pulses)	Iron:190 mT (85 mT) Air: 90 mT (85.4 mT)	MEPs	Iron core coil LI-rTMS ↑ MEP ratio vs sham
Tang et al. (2016b) 9-10 cells from 7 to 9 animals	Mouse, In vitro, motor and sensorimotor cortex slices - Motor and Sensorimotor Ctx	Healthy	1	iTBS		Electrophysiology: AP threshold, mean spike frequency, spike frequency, resting membrane potential	LI-rMS does not induce AP, but does hyperpolarise AP threshold and ↑ evoked spike-firing frequency. ↓ AP threshold, closer to the resting membrane potential. But the resting membrane potential and properties do not change.
Tang et al. (2018) *N* = 48; *n* = 16 per group	Mouse, C57Bl/6 J - Motor Ctx	Healthy	10	iTBS, Sham - 190s	120 mT	Single-Pellet Reaching Task, Western Blot (GluR1, GluR2, gephyrin), ELISA (BDNF)	Priming LI-rTMS: ↑ in skill accuracy, no change in rate of learning. Consolidation LI-rTMS: small ↑ in rate of learning, no change in skill accuracy. 24 h post stim: No change in GluR1, GluR2 and gephyrin. No change in BDNF.

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Table 2 (*continued*)

Study - N	Subject - **Stimulation Site**	Condition	Session No.	rTMS Parameters	Intensity (Intensity @ Target)	Measurements	Main Findings
Tang et al. (2021) $N = 15$; $n = 5$ per group	Mouse, Thy1-GFP-M - Motor Ctx	Healthy – Young Adult and Aged	1 or 4	iTBS (600 pulses) – 192s	120 mT (70 mT)	2-photon imaging *in vivo*; dendritic spine density, rate of spine loss/gain	Single session (young and aged, no group difference): ↓ dendritic spine density 45 h *pm*; ↑ rate of dendritic spine loss 21 h *pm*, ↓ rate of dendritic spine gain 45 h *pm* ↑ rate of dendritic spine gains at 21 h *pm*, only when single and multiple session animal data are pooled. Multiple sessions (only included young adults): ↑ rate of dendritic spine loss 21 h *pm*
Yang et al. (2019) $N = 36$; $n = 7–10$ per group	Rat, Wistar Rats - Not Reported	Healthy, Depression (CUS model)	14	1 Hz, Control - 1 h/day	20 mT	Sucrose test, elevated plus maze, morris water maze, local field potential and EPSPs for hippocampus, Western Blots for SYP, PSD95, NMDAR2B	Morris water maze: improved spatial learning with LI-rTMS. Reversal Probe trial: LI-rTMS increased quadrant dwell time Slopes of LTP potentiation phases improved closer to healthy controls in CUS + stim. Hippocampus Oscillation: Power density at delta and theta rhythms in CA1 and CA3 altered by treatment and model. PSD95 and NMDAR2B levels ↓ after CUS and increased in CUS + stim.
Ye et al., (2020) $n = 6$ per group	Human-derived, In vitro SH-SY5Y, neuroblastoma cell line	Healthy	1	Continuous 10 Hz (18,000 pulses), intermittent 10 Hz (1800 pulses), 1 Hz (1800 pulses), Control – 30 min	<10 mT	Cell culture medium collection (baseline, immediate *pms,* 1 h *pms,* 3 h *pms)* for colorimetric glutamate, GABA and calcium assays. Number of cells	Continuous 10 Hz: ↑ Ca^{2+} and ↑Glu concentration for at least 3 h *pms*. ↓ GABA conc at 1 h and 3 h *pms*. Intermittent 10 Hz: ↑ Ca^{2+} concentration only at 1 h and 3 h *pms*. ↑ Glu and ↓ GABA concentration only at 3 h *pms*. 1 Hz: gradual ↓ Ca^{2+} conc, only 3 h *pm* ↓ Glu concentration immediately *pms* only. ↑ GABA concentration 1 h and 3 h *pms*. No change in cell number.
Zong et al., (2020) $n = 8–10$ per group	Rat, Male Sprague Dawley Rats - Whole head	Ischemic Stroke	5	iTBS, Sham - 5 min/day	20 mT	Functional deficit behavioural tests. IHC: Dendritic spine, presynaptic, A1/A2, M1/M2, inflammation markers. TUNEL, NADPH oxidase activity and Superoxide Production Assay, Cytokine Array, Caspase Assay.	rTMS ↓ total infarct volume and improved functional deficits after 5 d post-stroke. rTMS-treated show preserved synaptic structure, less neuronal degeneration, markedly spared dendritic morphology. 21 d post-stroke: ↓ levels of elevated chemokines ↓. Microglia activation and shift toward M2 phenotype. ↓ pro-inflammatory cytokines. ↓ glial scar thickness. A1 to A2 switch in astrocyte phenotype. ↓oxidation, ↓ caspase-3 and caspase-9 activity, ↓TUNEL + cells suggest inhibited mitochondrial apoptotic pathway.

Abbreviations: AP, action potential; BDNF, brain derived neurotrophic factor; BHFS, biomimetic high frequency stimulation; BrdU, bromodeoxyuridine; Ca^{2+}, calcium; CAP, compound action potential; CC, corpus callosum; CRS, chronic restraint stress; CS56, chondroitin sulphate; cTBS, continuous theta burst stimulation; Ctx, cortex; CUS, chronic unpredictable stress; DARPP-32, dopamine- and cAMP-regulated phosphoprotein, Mr 32 kDa; dLGN, dorsolateral geniculate nucleus; DKO, double

knockout; dMRI, diffusion magnetic resonance imaging; GABA, gamma aminobutyric acid; EdU, 5-ethynyl-2′-deoxyuridine; GFAP, glial fibrillary acidic protein; Gln, glutamine; Glu, glutamate; glx, combined glutamate-glutamine concentration; GPIAS, gap prepulse inhibition of the acoustic startle reflex; HI-rTMS, high-intensity repetitive transcranial magnetic stimulation; IBA1, ionized calcium binding adaptor molecule 1; IHC, immunohistochemistry; IPA, interpretative phenomenological analysis; iTBS, intermittent theta burst stimulation; LC-MS/MS, liquid chromatography–mass spectrometry; LI-rTMS, low-intensity repetitive transcranial magnetic stimulation; M1, primary motor cortex; MEPs, motor evoked potentials; MGN, medial geniculate nucleus; MRS, magnetic resonance spectroscopy; mT, millitesla; NAc, nucleus accumbens; NADPH, nicotinamide adenine dinucleotide phosphate; NMDAR2B, N-methyl D-aspartate receptor subtype 2 B; nNOS, neuronal nitric oxide synthase; OL, oligodendrocyte; OLIG2, oligodendrocyte Transcription Factor 2; pms, post magnetic stimulation; PPI, pre-pulse inhibition; PrL, prelimbic cortex; PSD95, postsynaptic density protein 95; RGC, retinal ganglion cell; rs-fMRI, resting-state functional magnetic resonance imaging; RSN, resting state network; SC, superior colliculus; SYP, synaptophysin; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labelling; V2, secondary visual cortex; VEPs, visual evoked potentials; V-GLUT2; vesicular glutamate transporter 2; WT, wildtype.

30 min period there was an increase in Ca^{2+} levels only at 1 and 3 h post-stimulation. Meanwhile, 1 Hz stimulation was associated with a gradual decrease in Ca^{2+} concentration that reached significance versus controls only 3 h post stimulation (Ye et al., 2020). In contrast, live-cell ratiometric calcium imaging in primary astrocytes cultures demonstrated that 10 min of 1 Hz LI-rTMS, but not cTBS, 10 Hz or BHFS increased intracellular calcium levels in both the nuclear and cytoplasmic compartments (Clarke et al., 2017b). The calcium increase was evident within the first minute of 1 Hz stimulation in the astrocytes indicating rapid effects of stimulation and possibly an early role of calcium changes in LI-rTMS effects.

Both Grehl et al. (2015) and Clarke et al. (2017b) repeated their experiments with neurons and astrocytes respectively, in calcium-free medium and with thapsigargin treatment, which depletes internal stores of calcium. Only thapsigargin treatment attenuated the increase in Ca^{2+} concentration which indicates that LI-rTMS induces release of Ca^{2+} from intracellular stores (Grehl et al., 2015; Clarke et al., 2017b). These findings are similar to the conclusions of Luo et al. (2014) following PMF stimulations of neuronal cultures. Therefore, stimulation of calcium release from intracellular stores is likely a common mechanism of LI-rTMS induced change for both neurons and astrocytes. Since astrocytes are not electrically excitable, it may be that intracellular calcium changes are the main mechanism through which rTMS can influence glial activity.

3.1.3.2. Indirect measures. In addition to direct measurements of Ca^{2+} levels in cells, there are indirect methods of measuring changes in intracellular calcium dynamics that involve characterisation of calcium binding proteins such as calbindin and parvalbumin (PV) in postmortem tissue. Parvalbumin acts as a slow Ca^{2+} buffer in GABA-ergic neurons and can affect the amplitude and timing of internal calcium transients following an action potential which allows parvalbumin to potentially modulate short-term synaptic plasticity (Caillard et al., 2000). As a result, PV + densities are sometimes used as a measure of changes to intracellular calcium dynamics of inhibitory cortical neurons.

In a guinea pig model of tinnitus, 1 Hz stimulation and 10 Hz stimulation over the prefrontal cortex had significantly different effects on PV + densities in a dorsal region of the prefrontal cortex with an increase in PV + densities with 10 Hz stimulation compared to 1 Hz stimulation (Mulders et al., 2019). Changes in parvalbumin expression may modulate intracellular calcium dynamics of inhibitory cortical neurons, but the impact on excitability remains unclear. For example, following 10 Hz LI-rTMS in the tinnitus model, there was an increase in PV + cell density in the prefrontal cortex, and an increase in spontaneous firing rates in the medial geniculate nucleus. Since the prefrontal cortex is thought to modulate the MGN, the authors examined the relationship between these two measures at the level of individual animals, but results were not significantly correlated (Mulders et al., 2019). In contrast the density of cells expressing calbindin, a calcium binding protein similar to parvalbumin, did show a correlation with medial geniculate activity such that lower calbindin densities in the prefrontal cortex were associated with higher spontaneous medial geniculate activity (Mulders et al., 2019). Interestingly, the correlation was present even though the density of calbindin positive cells was not significantly altered following LI-rTMS. In summary, although there are parvalbumin changes in the cortex, they may not be associated with excitability changes in the downstream circuitry; similarly, the lack of significant LI-rTMS induced changes to calbindin density does not preclude an association with excitability changes in medial geniculate activity. While it is tempting to speculate that the increase in PV + density after stimulation may reflect LI-rTMS induced changes to intracellular calcium dynamics, functional studies of neuronal excitability are required to confirm this relationship and explore any functional implications.

Detecting Ca^{2+} -related changes after LI-rTMS is significant because Ca^{2+} is a powerful signalling molecule and triggers many pathways that can lead to long term changes in neuronal function, structure and connectivity. In the next section, we first describe evidence for biochemical changes within neurons that have been shown to occur after LI-rTMS. We then review the changes in connectivity that have been described in various models.

3.1.4. LI-rTMS – biochemical changes within neurons

3.1.4.1. Metabolites. LI-rTMS has been associated with changes to several metabolites. For example, in B50 rat neuroblastoma cells, an inhibitory neuron-like cell type, an *in vitro* assay assessed changes in 18 metabolites following a single session of LI-rMS and showed frequency-dependent changes (Hong et al., 2018). 1 Hz stimulation had stronger effects than 10 Hz stimulation, with more metabolites significantly affected (12 vs. 9) and greater fold changes following 1 Hz stimulation (Hong et al., 2018). Interestingly, metabolite concentrations were significantly decreased following LI-rTMS and this was suggested to be due to the increases in protein synthesis, intracellular calcium release and exocytosis which include mechanisms that have been reported following LI-rTMS (e.g. Grehl et al., 2015; Clarke et al., 2021).

The changes to metabolic activity of neurons following rTMS raise the possibility of changes to mitochondrial activity. Mitochondrial function underlies aspects of metabolic activity (McBride et al., 2006), synaptic plasticity (Tang and Zucker, 1997; Kang et al., 2008; Cai et al., 2011), neurotransmitter release (Billups and Forsythe, 2002; Kwon et al., 2016) and calcium buffering (Rizzuto et al., 2012), processes which are already implicated in LI-rTMS. Furthermore, the electron transfer components of the electron transport chain are ideal targets for electromagnetic stimulation as they involve charged molecules that could be sensitive to electromagnetic stimulation and proteins such as cytochromes which are sensitive to magnetic field effects (Katz et al., 2004). Changes to the electron transport chain could also impact other processes such as the Krebs cycle and lead to further downstream effects. Mitochondria are also highly sensitive to intracellular calcium levels and can also facilitate the release of internal calcium (Kann and Kovács, 2007), therefore mitochondrial changes induced by LI-rTMS may trigger the influx of internal calcium following stimulation. Further research to characterise mitochondrial changes may help fill in the gaps behind the initial stages of LI-rTMS changes.

3.1.4.2. Neurotransmitters. Among the cellular metabolites that are altered by LI-rTMS are molecules involved in neurotransmitter synthesis (Krebs cycle) (Hong et al., 2018), therefore it is no surprise that LI-rTMS can induce significant and immediate changes in neurotransmitter concentrations. However, results are inconsistent in the literature,

possibly due to the different methodologies used, which quantify neurotransmitter concentrations in different cellular spaces. For example, neurotransmitters quantified in whole brain, brain regions, or cell homogenates, as well as neurotransmitter quantification using MRS, cannot be attributed to a specific cellular location. However, analysis of the medium from cell culture experiments has been used to measure neurotransmitters located extracellularly or released from synapses. In future, using techniques like osmotic minipumps, which have not yet been applied to TMS models, may help to resolve the origin of neurotransmitter changes described below.

3.1.4.2.1. Glutamate. There is extensive evidence that LI-rTMS alters glutamate concentration, but results are complicated by the different protocols and measurement techniques that have been used. Effects have been described after a single session of LI-rTMS *in vitro*: Ye et al. (2020) analysed collected medium and saw an immediate decrease in extracellular glutamate concentration following 1 Hz stimulation, which recovered to baseline 1 h after stimulation. However, 10 Hz stimulation significantly increased extracellular glutamate concentration immediately after stimulation and continued to rise steadily at least 3 h after stimulation. In contrast, intermittent 10 Hz stimulation did not induce an immediate change in glutamate levels, but did show a significant increase at 3 h post stimulation. Therefore it may be that extracellular glutamate levels are modulated via two distinct mechanisms that work in the short-term (minutes) and long-term (hours) after stimulation.

Effects of chronic daily LI-rTMS were studied using MRS, with this technique allowing repeated measures to be taken from the same animals at weekly intervals. Glutamate levels were dependent on stimulation frequency, with strong but transient increases following 10 Hz stimulation and weak but longer lasting decreases following 1 Hz (Seewoo et al., 2019).

3.1.4.2.2. GABA. Acute and chronic changes following LI-rTMS are also seen for GABA concentrations. Acutely, Ye et al. (2020) saw an immediate increase in extracellular GABA concentrations compared to controls following one session of 1 Hz stimulation *in vitro* and concentrations continued to increase at 1 h and 3 h post-stimulation timepoints. In comparison, continuous, but not intermittent 10 Hz LI-rTMS led to a transient decrease in extracellular GABA concentration which returned to baseline by 3 h post stimulation.

Interestingly, following chronic stimulation in healthy rats, MRS data indicated that GABA levels are increased after 7 days of stimulation of 10 Hz stimulation, but not at 14 days of stimulation (Seewoo et al., 2019). It may be that following chronic excitatory LI-rTMS in healthy animals, a homeostatic mechanism increases GABA levels in the hours following stimulation. It is not clear why the increase in GABA no longer occurs after 14 days of stimulation, but it might be due to long term plasticity changes associated with repeated LI-rTMS.

Changes in GABA levels may also be dependent on properties of the circuitry being targeted (Rodger et al., 2012). In WT mice, GABA concentration in the superior colliculus was stable 2 h after a single session of BHFS (excitatory stimulation) delivered to visual cortex, but increased 24 h following stimulation, potentially reflecting a homeostatic response to the excitatory protocol (Rodger et al., 2012). In contrast, in ephrin-A2A5$^{-/-}$ mice, which have disorganised visual topography due to ectopic connections (described in more detail below, section 3.1.6.2), GABA concentration was significantly reduced 24 h after a single BHFS stimulation. The reason for the different response in normal and abnormal circuitry is unknown. One possibility is that the reduction in inhibitory neurotransmitter levels may contribute to the structural and functional reorganisation triggered by LI-rTMS in the abnormal visual pathway of ephrin-A2A5$^{-/-}$ mice (Rodger et al., 2012).

3.1.4.2.3. Monoamines. Unlike for glutamate and GABA, a single session of LI-rTMS does not alter the biosynthesis of monoamines. A single session of 10 Hz LI-rTMS *in vivo* demonstrated no significant changes to dopamine and serotonin metabolites, measured in homogenised brain samples. Liquid chromatography with tandem mass spectrometry of mouse brains following a single LI-rTMS session over the visual cortex showed no changes to serotonin, dopamine, their metabolites and amino-acid concentrations across several brain regions (Poh et al., 2019). Meanwhile, the same study showed that a single session of 10 Hz HI-rTMS induces changes in serotonin turnover, 3, 4-Dihydroxyphenylacetic acid (DOPAC) concentration, glutamate concentration and amino acid levels in the striatum or hippocampus (Poh et al., 2019). Changes to neuromodulators have been demonstrated with HI-rTMS across several occasions (for review see Funke and Benali, 2011; Lenz and Vlachos, 2016; Moretti et al., 2020). In this case, the changes seen in HI-rTMS but not LI-rTMS highlight the lower impact that LI-rTMS likely has compared to HI-rTMS, and that multiple stimulations may be required to identify *in vivo* changes with LI-rTMS.

In summary, changes in GABA and glutamate levels are congruent with the idea that LI-rTMS can influence neuronal excitability and potentially plasticity. In addition, serotonin and dopaminergic changes may further modulate neuronal responses but may require extended stimulation conditions, or higher intensity magnetic fields. Changes to neurotransmitter release induced by rTMS have important implications for brain-wide changes since changes in cell communication can have indirect effects on other cells and regions that spread beyond directly stimulated neurons.

3.1.5. LI-rTMS alters connectivity

3.1.5.1. Functional connectivity. Changing the activity and function of one area can have widespread effects on the downstream (and potentially upstream) communication with other areas of the brain. Indeed, studies using fMRI have found that LI-rTMS can alter resting-state networks (synchrony between brain regions) after both single and multiple stimulations in a manner similar to changes seen after HI-rTMS in humans (Seewoo et al., 2018, 2019). Changes to resting-state network activity were dependent on frequency, and could be induced unilaterally or bilaterally, or within specific networks. For example, analysis of resting-state activity in the default-mode network showed that a single stimulation of 1 Hz LI-rTMS caused bilateral decreases in synchrony, with greater contralateral changes, whereas 10 Hz, BHFS and cTBS stimulation caused mostly ipsilateral changes with excitatory protocols demonstrating increased synchrony and cTBS showing decreased synchrony (Seewoo et al., 2018). Furthermore in a follow-up study assessing 14 days of LI-rTMS, 10 Hz stimulation increased functional connectivity in the DMN, the cortico-striatal-thalamic network and the basal-ganglia network whereas 1 Hz stimulation attenuated functional connectivity in the cortico-striatal-thalamic network, basal-ganglia network and salience network (Seewoo et al., 2019). Therefore, network connectivity data shows that not only do different frequencies increase or decrease synchrony, they also have effects that are network specific.

The frequency of LI-rTMS can also influence the timing and duration of effects. This is particularly relevant for understanding the potential latency and longevity of therapeutic rTMS. Seewoo et al. (2019) demonstrated that 14 days of LI-rTMS caused sustained changes to functional connectivity that persisted following cessation of stimulation. However, the timecourses of 10 Hz and 1 Hz effects were very different: 10 Hz effects were stronger and developed more quickly than those of 1 Hz, but returned to baseline within a week of stimulation cessation. In comparison, 1 Hz stimulation resulted in relatively subtle but sustained changes for at least two weeks after the last stimulation session. Therefore, manipulating the frequency of LI-rTMS frequency has enormous potential to target not only specific brain regions and their connectivity, but also the timing and duration of therapeutic effects.

3.1.5.2. White matter changes. While resting state fMRI has been useful in identifying changes in connectivity between different brain regions, it remains unclear what causes these changes. Neurotransmitter release is likely involved (section 3.1.4.2), but changes in the white matter, in

particular to myelination can have significant effects on whole-brain activity. Here we review the evidence for LI-rTMS altering white matter using data from magnetic resonance diffusion imaging, as well as cellular studies of oligodendrocytes, which are the cells that myelinate CNS axons.

3.1.5.2.1. LI-rTMS and diffusion MRI. Using diffusion tensor imaging, 10 Hz and 1 Hz stimulation over 15 days were shown to increase fractional anisotropy at 1 week and 2 weeks post stimulation, respectively (Seewoo et al., 2021). The increase in fractional anisotropy likely reflects an increase in "white matter integrity". Seewoo et al. (2021) also assessed kurtosis measures which reflect tissue complexity by quantifying the degree of restriction of water diffusion, either perpendicular (radial kurtosis) or parallel (axial kurtosis) to the axonal direction. Chronic 10 Hz stimulation changed kurtosis measures across several regions, while 1 Hz stimulation showed only a decrease in radial kurtosis within the corpus callosum. Changes in kurtosis could be due to changes in the microstructure of white matter, such as increased myelination, increased number of axons and neurites, or changes to intracellular structures. Chronic 10 Hz LI-rTMS was also able to rescue delayed brain maturation-related changes in a chronic restraint model of depression in rats and was associated with greater myelin protein density following immunohistochemistry analyses which suggest increased myelination is a mechanism for the changes in white matter.

3.1.5.2.2. LI-rTMS and oligodendrocytes. Notably, the diffusion MRI data suggesting increased myelination are supported by recent studies showing that chronic LI-rTMS promotes the survival of premyelinating oligodendrocytes (Cullen et al., 2019, 2021). Fourteen days of excitatory LI-rTMS using an iTBS protocol increased survival of premyelinating oligodendrocytes in the motor and visual cortices, without changing oligodendrogenesis (Cullen et al., 2019). It appears that low intensity iTBS promotes the survival of immature oligodendrocytes through reduced apoptosis, which results in more newly born oligodendrocytes reaching maturity (possible mechanisms behind pro-survival effects of LI-rTMS are discussed in section 3.2). This pro-survival effect required 28 consecutive days of stimulation (14 days had no effect), presumably because this is the time required to support premyelinating oligodendrocytes to the end of their maturation. However, LI-rTMS effects did not outlast the stimulation period. Pre-myelinating oligodendrocyte survival mostly returned to baseline by 7 days post-stimulation and at 14 days post-stimulation all regions had returned to control levels (Cullen et al., 2019). The ability of LI-rTMS to improve oligodendrocyte survival has important implications for the possible treatment of demyelinating diseases such as Multiple Sclerosis where impaired survival and maturation of oligodendrocytes is a key factor.

In addition to increasing oligodendrocyte survival, LI-rTMS can modulate myelin sheath plasticity which alters the conduction velocity of axons along the corpus callosum (Cullen et al., 2021). Mice treated with iTBS showed a significant increase in periaxonal space and reduced node length, which resulted in reduced conduction velocity along the axon. iTBS-treated mice also showed a 40% increase in compound action potentials recorded from *ex vivo* field potential recordings. These changes were only seen in myelinated axons, unmyelinated axons showed no difference between iTBS and sham-stimulated mice. It may be that iTBS-induced plasticity of oligodendrocytes and subsequent lowered conduction velocity results in more action potentials arriving simultaneously, reflected by the larger amplitude of the recorded compound action potential (Cullen et al., 2021). Although these oligodendrocyte plasticity changes were not associated with improvements in motor coordination (Cullen et al., 2021), they may have contributed to improved fine-motor-skills learning reported in a previous study (Tang et al., 2018).

Overall, these changes to white matter structure, myelination and conductance velocity after LI-rTMS may contribute to the network level effects described in section 3.1.5. Increasing synchrony of action potential arrival times could increase probabilities of post-synaptic firing and increased neuronal activity could feedback to further promote myelination (Barres and Raff, 1993; Cullen et al., 2021). Changing conduction velocity and synchrony can also have broader effects: changes to spike-timing can alter whether potentiation or depression is induced in the brain (Feldman, 2012; Markram et al., 2012) and altering firing frequency could also influence brain wave oscillation. Together these changes could contribute to the changes in functional connectivity seen following LI-rTMS using MRI techniques.

3.1.6. LI-rTMS modulation of excitability mediates behavioural change

Although LI-rTMS has been shown to alter various aspects of cellular and network functioning, behavioural changes remain the most clinically and translationally relevant outcomes. Here we summarise the behavioural evidence that LI-rTMS induces neuroplasticity and in some cases these studies show that changes to plasticity and cortical activity are associated with beneficial changes to learning, tinnitus, and activity-driven re-organisation of the cortex in mice with abnormal brains and depression.

3.1.6.1. Learning. Learning and memory are key behavioural outcomes associated with plasticity and have been extensively studied with a wide range of neuromodulation techniques. To date, there is evidence that LI-rTMS may have benefits in motor and spatial learning paradigms.

In a motor learning task, multiple sessions of excitatory LI-rTMS over the motor cortex improved performance in mice in a timing-dependent manner. Priming stimulation, or stimulation before each training session, significantly increased skill accuracy compared to sham stimulation, but there was no change in rate of learning versus sham. However, consolidation stimulation - stimulation immediately after each training session - showed the reverse with a small increase in rate of learning compared with sham controls, but no significant difference in skill accuracy (Tang et al., 2018).

Priming stimulation improvements are thought to be mediated by LI-rTMS enhancing LTP processes, while consolidation effects may act on later aspects of LTP such as structural plasticity of dendrites (Tang et al., 2018) In an effort to gain insight into the potential mechanisms of LI-rTMS in this learning paradigm, protein expression of GluR1, GluR2 and gephyrin protein was investigated in these mice as they are markers of synaptic plasticity linked to AMPA receptor expression (GluR1, GluR2) and plasticity at the inhibitory synapse (gephryn). However, no changes were found - possibly because protein levels were measured postmortem several weeks after chronic stimulation had been completed. Nonetheless, the difference in behavioural results for priming versus consolidation stimulation emphasises the importance of brain state during stimulation and suggests that stimulation interacts with endogenous brain activity via multiple mechanisms which can lead to different functional outcomes.

In a different paradigm, stimulation over the cerebellum with excitatory LI-rTMS increased spatial memory but not spatial learning in the Morris water-maze task compared to sham-treated mice (Morellini et al., 2015). In addition, mice that received LI-rTMS showed evidence of structural plasticity, with Purkinje cells having dendritic arbours that were longer, with greater surface area and greater spine density compared to sham mice. Unfortunately, it was not possible to determine whether the structural change was a direct consequence of LI-rTMS, or secondary to the improvement in memory over time, since similar changes to Purkinje cell dendritic morphology were reported following motor learning (Lee et al., 2007). Interestingly, in a mouse study of learning and reversal learning tasks in which no behavioural change was induced by LI-rTMS, hippocampal dendritic spine density (detected by Golgi staining) was not significantly altered, even though mice successfully learned the task (Sykes et al., 2013).

Further addressing the relationship between rTMS and morphological plasticity, Tang et al. (2021) used two-photon imaging to track dendritic spine plasticity in the motor cortex of both young adult and aged mice in the motor cortex. The study found that a single session of

LI-rTMS using an iTBS protocol over the motor cortex reduced dendritic spine density at 45 h post stimulation. The rate of dendritic spine loss was increased at a 21 h post-stimulation timepoint and rate of spine gain was decreased at 45 h post-stimulation. Young adult mice also underwent multiple sessions of LI-rTMS and showed an increase in rate of dendritic spine loss 21 h following the final stimulation, however there was no change at 45 h post stimulation in contrast to the longer-lasting changes seen with a single stimulation. The lack of a cumulative or longer lasting effect of multiple stimulation sessions is surprising, given the assumption that multiple stimulation sessions of HI-rTMS are required for long-term therapeutic effects, and highlights the need to characterise the timeline of various LI-rTMS mechanisms.

Although Tang et al. (2021) did not assess a functional outcome in this study, their previous work has shown that LI-rTMS using iTBS can improve motor learning (Tang et al., 2018), and others have shown that changes in spine density facilitate learning of skilled motor behaviours (Xu et al., 2009; Fu et al., 2012). Taken together, these studies strongly suggest structural synaptic plasticity as a key mechanism underlying LI-rTMS-induced changes to neural plasticity and connectivity that underpin learning (Tang et al., 2021). Interestingly, response to a single stimulation with LI-iTBS was the same for both the young adult and aged animals, which is promising as older adults show reduced HI-rTMS induced plasticity (Todd et al., 2010; Opie et al., 2017) and synaptic plasticity is often impaired with age (Landfield et al., 1978; Pinho et al., 2017; Davidson et al., 2020).

3.1.6.2. *LI-rTMS in ephrin-A knockout mice: modelling abnormal circuitry*

3.1.6.2.1. Measuring plasticity in visual circuits. The strength and stability of neuronal connections are often tied to neuronal activity, as per the Hebbian theory of plasticity - neurons that fire together wire together (Hebb, 1949). This concept underpins studies that have used LI-rTMS with the goal to increasing electrical activity within abnormal brain circuits and drive reorganisation. The model system used in these studies is a genetically modified mouse strain that lacks *ephrin-A2* and *ephrin A5*. Ephrins are cell-surface axon guidance molecules and ephrin-A2 and ephrin-A5 proteins are crucial in mapping topography within the visual pathway (Triplett and Feldheim, 2012). Mice that are double knockout for these genes (ephrin-A2A5$^{-/-}$) have abnormal connectivity in the visual system in the form of ectopic connections between the retina, lateral geniculate nucleus (LGN) and visual cortex (Feldheim et al., 2000), as well as visuomotor behavioural deficits (Haustead et al., 2008). Applying LI-rTMS in this model has led to a number of insights into how stimulation interacts with endogenous brain activity to facilitate reorganisation of disordered neural circuits.

In a first series of experiments, chronic LI-rTMS was shown to reduce the number of ectopic retinocollicular terminals (Rodger et al., 2012), shift disordered corticotectal projections closer to appropriate locations, and reduce abnormally high dispersion of retrogradely labelled dLGN neurons in ephrin-A2A5$^{-/-}$ mice (Makowiecki et al., 2014; Poh et al., 2018). Furthermore, these changes in connectivity were associated with the restoration of normal visual tracking behaviour (Rodger et al., 2012; Poh et al., 2018). Subsequent experiments attempted to boost Hebbian plasticity by increasing visual activity during LI-rTMS, but surprisingly reorganisation did not occur when mice were performing a concurrent visual discrimination task during stimulation (Poh et al., 2018). Taken together, these experiments suggest that topographical reorganisation is caused by induced currents from LI-rTMS that may activate weak, ineffective or silent ectopic synapses and promote the elimination or shift of inappropriate synapses (Rodger et al., 2012). However, it may be that very high levels of activity in visual brain regions as a result of concurrent visual and attentional stimuli result in both abnormal and normal projections being reinforced (Poh et al., 2018). Interestingly, chronic LI-rTMS is effective in beneficially reorganising disordered neural circuits, but changes to visual behaviour or topographical organisation were not seen in wildtype mice (Rodger et al., 2012). The implication is that LI-rTMS may specifically target abnormal connectivity, without disrupting normal connectivity and function. These data suggest that neuromodulation as an adjuvant for rehabilitation therapies could be safe and effective, but it will be important to understand how stimulation interacts with endogenous brain activity to avoid over-stimulation and determine an optimal dosing protocol.

Ephrin-A2A5$^{-/-}$ mice have also been used to explore the influence of acute and chronic LI-rTMS on synaptic function using electrophysiological recordings of visually evoked potentials (VEP). Characteristics of VEPs recorded from visual cortex show that ephrin-A2A5$^{-/-}$ mice have smaller VEP amplitudes compared to wildtype mice, suggesting a less coherent population response (Makowiecki et al., 2018). Interestingly, a single session of LI-rTMS altered VEP properties, but these effects required evoked activity during stimulation (Makowiecki et al., 2018). These results support findings from *in vivo* studies described above that neural activity and the degree of coordination across neuronal populations interact with LI-rTMS to alter excitability in a context-dependent manner. Extending these findings to chronic LI-rTMS, field recordings of visually evoked responses from the superior colliculus in ephrin-A2A5$^{-/-}$ mice show that 14 days of daily LI-rTMS increased receptive field size compared to controls (Rodger et al., 2012). These data are reminiscent of classical studies of optic nerve regeneration in fish and visual system development in mammals, showing altered receptive field properties when visual activity is manipulated through chemical or environmental interventions (Eisele and Schmidt, 1988; Thornton et al., 1996; Leu and Schmidt, 2008). Furthermore, investigation of candidate gene expression in ephrin-A2A5$^{-/-}$ mice implicates similar molecular changes, including an increase in brain-derived neurotrophic factor (BDNF) and neuronal nitric oxide synthase (nNOS) levels (Rodger et al., 2012; Makowiecki et al., 2014). These classical visual system plasticity mechanisms facilitate neuronal responses and enhance neuronal synchrony and may underpin some of the plasticity effects of acute and chronic LI-rTMS.

3.1.6.2.2. LI-rTMS impact on motivation and attentional behaviours. Although ephrin-A2A5$^{-/-}$ mice are best characterised for their visual deficits, other brain regions are affected by the lack of ephrin proteins, and of particular interest to LI-rTMS studies are changes to the dopaminergic system (Halladay et al., 2004; Sieber et al., 2004; Cooper et al., 2009; Yates et al., 2014; Sheleg et al., 2016). Although behaviour related to dopamine neurotransmission has not been fully characterised in ephrin-A2A5$^{-/-}$ mice, there is evidence that these mice may have abnormalities in attention and motivation, and in some cases these have been rescued by LI-rTMS. The first evidence come from a study that used a visual learning task to increase visual activity during LI-rTMS (see section 3.1.6.2.1 above). An unexpected finding of this study was that ephrin-A2A5$^{-/-}$ mice completed significantly fewer trials than the wildtype mice, and this behaviour was rescued by LI-rTMS (Poh et al., 2018). Because learning and accuracy were not affected, it was hypothesised that ephrin-A2A5$^{-/-}$ might have abnormal attention and/or motivation. A follow-up study (Moretti et al., 2021) using progressive ratio testing in an operant chamber did not detect any changes in motivation in ephrin-A2A5$^{-/-}$ mice, but did suggest increased persistence (see also Arnall et al., 2010) although this behaviour was not affected by LI-rTMS (Moretti et al., 2021). Future avenues for investigation include further characterisation of behaviours associated with dopamine in ephrin-A2A5$^{-/-}$ mice, and investigation of potential links between locomotion, exercise and task engagement.

3.1.7. *LI-rTMS in clinical models*

3.1.7.1. Tinnitus. Tinnitus is a disorder that involves the perception of a phantom auditory sensation (ringing or buzzing sound) and is thought to be associated with hearing loss and resulting hyperactivity. Clinical trials in humans suggest that HI-rTMS can improve symptoms although it remains unclear which protocols and brain targets are the most

effective. A series of studies in a guinea pig model of hearing loss have explored stimulation intensity, frequency and location, and show that LI-rTMS reduces the perception of tinnitus and may alter excitability in specific regions within the auditory pathway. Delivering 1 Hz LI-rTMS over the auditory cortex contralateral to the hearing loss for 10 days reduced the perception of tinnitus, possibly by increasing long term depression and reducing overall activity in the auditory cortex (Mulders et al., 2016). However, when stimulation was delivered to the contralateral prefrontal cortex, neither 1 Hz nor 10 Hz reduced the behavioural signs of tinnitus, even though spontaneous activity in the medial geniculate nucleus and density of calcium binding proteins were altered (Mulders et al., 2019). For further context, when HI-rTMS was applied to the prefrontal cortex in the same guinea pig model, both 20 Hz and iTBS frequencies reduced the perception of tinnitus, with each frequency appearing to do so via different mechanisms (Zimdahl et al., 2021). However one consideration is that electric field modelling in Zimdahl et al. (2021) demonstrates that areas outside the prefrontal cortex, including the auditory cortex, still receive stimulation at lower intensities. Therefore, the influence of peripheral lower intensity fields from HI-rTMS on the auditory or other cortices cannot be excluded. Therefore, although there are clear cellular effects with different LI-rTMS frequencies, choosing appropriate intensities and stimulation targets appears to be key for optimal functional and behavioural outcomes.

3.1.7.2. Depression. rTMS at high intensity has various potential clinical applications and is most commonly used as a treatment for depression (Fitzgerald, 2020; Lefaucheur et al., 2020). However there is increasing evidence that stimulation at lower intensity may be as effective as high intensity. In some cases LI-rTMS also provide additional benefits not obtained with high intensity due to recruitment of different neurobiological mechanisms.

The earliest studies of low intensity stimulation showed that non-focal PMF stimulation in humans resulted in significant improvements in mood compared to sham groups in patients with bipolar depression (Rohan et al., 2004, 2014) or major depression (Martiny et al., 2010; Rohan et al., 2014). PMF did not alter mood in healthy controls and unmedicated patients reported slightly greater benefits of stimulation on mood. In one study, mood improved immediately following stimulation (Rohan et al., 2014), while another required at least 2 weeks of daily stimulation before improvement was significant compared to sham (Martiny et al., 2010). In addition to the low intensity and lack of focality a further difference of these studies with conventional rTMS used to treat depression is the use of BHFS that are not currently in use in the clinic. Nonetheless, these PMF studies provide evidence that non-focal low-intensity electromagnetic stimulation can improve depression symptoms.

Preclinical studies have confirmed a biological effect of PMF in rodent models of mood disorders. A recent study in rats subjected to the chronic unpredictable mild stress (CUS) model of depression used a PMF style coil (non-focal, Hemholtz coil) for an hour per day for 14 days, but used 1 Hz stimulation, which is a common rTMS frequency (Yang et al., 2019). Measures of anxiety- and depression-like behaviours assessed by the elevated plus maze, sucrose test or Morris water maze test, did not improve following stimulation. Reversal learning assessed using the Morris water maze did appear to improve following stimulation, but the effect was seen in both depressed and control mice. However, a key difference was detected when using electrical stimulation to induce LTP in the hippocampus. The induced potentiation was significantly reduced in CUS model of depression, whereas CUS rats that received PMF showed normal levels of potentiation. Local field potential recordings in the hippocampus also showed alterations in neural activity that suggested that PMF regulated neural oscillations in the hippocampus for both CUS and control rats. Supporting these physiological changes, PMF stimulation restored protein levels of PSD95 and NMDAR 2 B (also known as GluN2B), which were down regulated in CUS rats (Yang et al., 2019). The return of NMDAR protein close to control levels following PMF supports the change in LTP seen in the hippocampal recordings as NMDAR is important for LTP induction. Overall, these results support the notion that low-intensity stimulation is effective primarily in abnormal circuits, with stimulation-induced protein and electrophysiological changes seen only in the rat model of depression.

More recently, a range of rTMS intensities were tested using focal, unidirectional coils in an olfactory bulbectomy model of depression in mice (Heath et al., 2018). Heath et al. (2018) assessed three intensities of stimulation – 12 mT, 90 mT and 1.2 T (HI-rTMS), at 10 Hz for 3 min (1800 pulses) across 20 daily sessions. Interestingly it was found that the 90 mT and 1.2 T conditions, but not the 12 mT condition, had significant effects on behaviours that are relevant for modelling depression in humans (Heath et al., 2018). Both 90 mT LI-rTMS (termed medium intensity: MI-rTMS in the article) and HI-rTMS reduced psychomotor agitation in the forced swim test, a characteristic of the olfactory bulbectomy model of depression. However only 90 mT LI-rTMS showed a significant increase in BDNF levels in the frontal cortex and hippocampus, accompanied by increased neurogenesis in the dentate gyrus (Heath et al., 2018). It may be that increased BDNF drives the increase in neurogenesis, which is considered to be an anti-depressant effect. Since HI-rTMS did not alter BDNF or neurogenesis, LI-rTMS may have mechanisms of action that are distinct from those HI-rTMS, and which have the potential to provide longer-lasting effects as increased neurogenesis suggests long-term structural changes.

Heath et al. (2018) was the first to systematically compare different rTMS intensities while maintaining other parameters such as frequency and coil focality as similar as possible (although HI-rTMS focality could not match the smaller coils). Their results highlight an important consideration that higher intensity stimulations may not always be better as 90 mT LI-rTMS not only matched anti-depressant effects of high intensity stimulation, but also appears to contribute its own beneficial effects not seen with higher intensity stimulation.

3.1.7.3. Stroke and injury. In addition to the effects on neuroplasticity that have been the focus of the previous sections, LI-rTMS has demonstrated neuroprotective and reinnervation effects. Several studies looking at LI-rTMS have used stroke and injury models to help understand how rTMS may attenuate the brain's response to injury and/or improve recovery following an insult. Discussion of these models and the possible mechanisms of the neuroprotective LI-rTMS effects are covered in detail in section 3.2.

3.2. Neuroprotective functions of LI-rTMS

3.2.1. Increase in BDNF

BDNF is an important growth factor that is implicated in plasticity, repair and regeneration of CNS neurons and the goal of many therapeutic interventions is to increase BDNF levels in the brain. BDNF is often, but not always, shown to be upregulated following LI-rTMS. For example, even in the absence of a lesion, BDNF levels in healthy wild-type and ephrin-A2A5$^{-/-}$ mice are higher in the superior colliculus and visual cortex following stimulation (respectively: Rodger et al., 2012; Makowiecki et al., 2014) and is associated with reorganisation of abnormal connections. However, whether upregulation occurs after a single session of LI-rTMS or requires multiple sessions varies between studies, and may depend on the brain region targeted (Rodger et al., 2012; Makowiecki et al., 2014). BDNF is also increased in a mouse model of depression following LI-rTMS, but not HI-rTMS which may contribute to increased neurogenesis seen in the same study (Heath et al., 2018). In both of these models, upregulation of BDNF is associated with plastic changes that lead to behavioural improvements – in vision (Rodger et al., 2012) and in behaviours associated with depression and anxiety (Heath et al., 2018).

LI-rTMS can also induce regeneration in *in vivo* and *ex vivo* models of cerebellar lesions which appears to be mediated by upregulated BDNF expression. Two weeks of daily LI-rTMS (iTBS, BHFS and 10 Hz, but not 1 Hz or cTBS) over lesioned explants and in mice with unilateral olivocerebellar lesion has been found to increase Purkinje cell reinnervation when compared with sham stimulation (BHFS only: Morellini et al., 2015; BHFS, iTBS, 10 Hz, cTBS, 1 Hz: Dufor et al., 2019). The ability for LI-rTMS to induce regeneration appears to be mediated by BDNF, as adding exogenous BDNF, which on its own can induce reinnervation, had no additive reinnervation effects compared to LI-rTMS alone (Morellini et al., 2015). Therefore, excitatory LI-rTMS may upregulate BDNF pathways, resulting in reinnervation. Indeed, BDNF mRNA expression in lesioned cerebellar explants is greater following excitatory LI-rTMS compared with inhibitory LI-rTMS (Grehl et al., 2016).

Interestingly, other studies that have measured BDNF after chronic LI-rTMS have found no change, including in an optic nerve crush model (Tang et al., 2015), a guinea pig tinnitus model (Mulders et al., 2016), and after 10 days of motor learning (Tang et al., 2018). Notably, in the optic nerve crush model, stimulation over the operated eye did not improve nerve regeneration, with no changes to retinal ganglion cell survival, or axon regeneration, suggesting that not all brain regions or types of injury are susceptible to LI-rTMS (Tang et al., 2015).

3.2.1.1. Mechanisms of BDNF upregulation. Regulation of BDNF levels is notoriously complex (for review see Brigadski and Leβmann, 2020), with multiple transcriptional control sites and mechanisms at the gene transcription level, and additional post-translational regulation through immature protein forms (e.g. Mowla et al., 2001). Although there is evidence that LI-rTMS upregulates BDNF expression at both the mRNA and protein levels, the mechanisms have not been investigated. Since BDNF expression is activity dependent, it could be that an increase in neuronal activity induced by LI-rTMS (Tang et al., 2016a) leads to increased levels of BDNF. BDNF is also mediated by internal Ca^{2+} release (Kolarow et al., 2007) so LI-rTMS-induced release of intracellular calcium stores (Grehl et al., 2015; Ye et al., 2020) could be an alternate or additional mechanism behind BDNF upregulation. Dissecting the triggers for BDNF upregulation is challenging because calcium levels and neuronal activity are closely intertwined, and the chronological and causal sequence of molecular and cellular events immediately following LI-rTMS is not fully characterised. On a longer time scale, upregulated BDNF, while playing a role in neuronal survival, growth and plasticity, also modulates several downstream signalling pathways, one of which is the PLC/DAG/IP3 pathway, which itself can increase intracellular calcium concentrations (Bathina and Das, 2015). It will be important in future to dissect out the timing and relative contributions of these important pathways to LI-rTMS facilitation of reinnervation, regeneration and synaptic plasticity.

3.2.2. Reduced inflammatory response – a role for non-neuronal cells

As their name suggests, neuromodulatory techniques alter neuronal biology, but an unexpected outcome of LI-rTMS is modulation of the brain's inflammatory response to injury, implicating non-neuronal cells as potential targets of stimulation. Although it is still not clear how much LI-rTMS affects glial cells directly or indirectly as a result of neuronal changes, there is evidence *in vitro* in pure astrocyte cultures that LI-rTMS directly alters calcium levels in astrocytes, and alters the expression of genes involved in inflammation (see below section 3.2.3 for review of genetic changes).

Glial changes following LI-rTMS have also been demonstrated in *in vivo* and *in vitro* injury models, with changes to the glial scar (Clarke et al., 2017a; Zong et al., 2020), as well as to glial phenotype and morphology (Clarke et al., 2017a; Zong et al., 2020). For example, after a cortical stab injury in mice LI-rTMS induced changes in glial cell density proximal to the lesion, including changes in astrocytic and microglia densities (Clarke et al., 2017a). However, these effects differed depending on the age and gender of the animals (Clarke et al., 2017a). This suggests that interaction of age and gender with LI-rTMS is an important factor, although it has not been studied extensively in the literature. In a model of photothrombotic ischemic stroke in mice, glia scar thickness was reduced in LI-rTMS treated mice compared to controls. Microglia phenotype was also affected in comparison to sham animals, with an apparent shift towards activation of M2 phenotypes, which have neuroprotective properties (Zong et al., 2020). Similarly, in astrocytes, a stroke-induced increase in 'neurotoxic' A1 phenotype was attenuated by rTMS and 'protective' A2 phenotype levels were increased with rTMS (Zong et al., 2020). In another study, astrocytic swelling was also transiently reduced after an *in vitro* scratch assay with 1 Hz stimulation, however this effect was gone by 48hrs post-stimulation and specific to 1 Hz stimulation (Clarke et al., 2017b).

In vivo studies have attempted to link glial changes after LI-rTMS to functional improvement. Using a hybrid of PMF (Helmholtz coil) and LI-rTMS (iTBS stimulation protocol and 20 mT intensity), a model of photothrombotic ischemic stroke in mice showed evidence of reduced damage following 5 min of stimulation delivered daily for 5 days following the stroke (Zong et al., 2020). Total infarct volume was reduced by 45.33% in rTMS-treated stroke animals and behavioural tests assessing functional deficits (cylinder, adhesive, ladder dexterity and hanging wire tests) showed that at 5 days post-stroke LI-rTMS--treated mice performed significantly better than mice receiving sham rTMS (Zong et al., 2020). On a cellular level there was evidence of reduced cell death and oxidative stress as well as sparing of synaptic and dendritic morphology. Furthermore, at 21 days post stroke, rTMS showed suppressed cytokine and chemokine levels vs. sham (Zong et al., 2020). These changes could be associated with increased cell survival, possibly via mitochondrial changes: rTMS inhibited the mitochondrial apoptotic pathway, with reduced TUNEL + cells, and caspase-3 and caspase-9 activity. Various markers of oxidative stress, which are linked to mitochondrial activity and damage were also attenuated following LI-rTMS. In contrast, a medial cerebral artery occlusion stroke model in rats showed no effect of LI-rTMS at any of the 3 frequencies studied (1 Hz, 10 Hz and BHFS) on glial scarring and no improvement in infarct volume, neuronal survival or function (Bates et al., 2012). However, BHFS and 1 Hz stimulation increased macrophage infiltration suggesting a reduction in inflammatory response, consistent with the studies discussed above.

Another approach to studying LI-rTMS after injury has been to screen for candidate genes involved in neuronal regrowth and reinnervation. In an *in vitro* lesioned explant model of reinnervation Grehl et al. (2016) reported changes to mRNA expression of BDNF, Pax3, Sia2 and Sia4 after a single stimulation at either 1 Hz, 10 Hz or BHFS (Grehl et al., 2016). More changes in the cerebellum *ex vivo* following reinnervation were reported by Dufor et al. (2019), where various genes associated with neurotrophic signalling were examined. Ten genes showed significant changes in gene expression, with 12 other mRNAs indicating a likely change (Dufor et al., 2019). iTBS and BHFS, the two frequencies that induced reinnervation, increased gene expressions related to neurogenesis and response to axon injury, as well as genes whose products are linked with post-lesion reinnervation in other neural systems (β_2-microglobulin, β -Glucuronidase, and neurotrophin-3/NT3) (Dufor et al., 2019).

Overall, experiments using a wide range of injury stroke models provide strong evidence that in addition to upregulating genes associated with regeneration and plasticity, LI-rTMS also reduces inflammatory response and cell death via altered glial and mitochondrial activity. Both glia and mitochondria are sensitive to changes in calcium which is likely how LI-rTMS induces these effects. Although there is some evidence that magnetic and electric field effects act directly on mitochondria (e.g. Katz et al., 2004; Calabrò et al., 2013b, 2013a), further investigations targeting mitochondrial activity could help elucidate these changes. A weakness in the animal models of LI-rTMS stroke literature is that there are no studies of the excitability of

intrahemispheric pathways, which is the mechanism believed to underpin benefits of rTMS in stroke patients (but see Carson, 2020). This is likely because the rodent coils that are available lack the combination of high focality and high intensity required to elicit changes in excitability reported in human patients. Nonetheless, there is evidence that LI-rTMS rodent coils with higher intensity capability are focal enough to stimulate one hemisphere, and can cause changes in cortical excitability (Tang et al., 2016a, 2016b) suggesting that these experiments would be valuable.

3.2.3. Genetic changes

Although no full transcriptomic analyses following LI-rTMS has been carried out, there have been PCR array studies carried out in defined cell populations *in vitro* in order to differentiate between the different cell types. These studies - although limited in the number of genes studied - provide an indication of the breadth of LI-rTMS effects.

In cortical neurons (enriched in inhibitory neuronal subtypes), a 90 gene array study was carried out, focussing on calcium signalling-related genes. LI-rTMS induced gene changes include genes involved in regulating cell survival, apoptosis, inflammation, signalling, plasticity or cell structure/morphology. Genetic changes are also frequency-dependent, although overlap across frequencies can occur and appear to have functional outcomes. For example, significant gene changes were seen following a single stimulation of neurons *in vitro* using 1 Hz, 10 Hz, and BHFS protocols (Grehl et al., 2015). The majority of the regulated genes were associated with either cell survival and apoptosis, or cell morphology and migration. At the end of four daily stimulations there was an increase in apoptotic cells with 10 Hz and 100 Hz stimulation, and impaired cell survival of excitatory neurons after 1 Hz stimulation (Grehl et al., 2015). Therefore, regulation of neuronal survival and apoptosis genes by LI-rTMS may contribute to an increase in cell death *in vitro*. Interestingly, no evidence of neuronal death has been described *in vivo*, suggesting that it is important to keep in mind the limitations of cell culture when interpreting these results. Neuronal cultures often lack features present *in vivo* such as glia, other supporting cells as well as lamination or other complex organisation. There are also various differences influencing function between *in vivo* and *in vitro* models such as the number of synaptic connections, spontaneous activity levels and absence of neuromodulatory inputs from other regions (e.g. Belle et al., 2018). As a result, although useful to investigate basic mechanisms, *in vitro* systems may not fully reflect *in vivo* function.

In astrocytes, Clarke et al. (2021) investigated LI-rTMS induced gene changes with follow up analysis of protein expression. Clarke et al. (2021) looked at 125 astrocyte-related genes in purified astrocytic cultures and found that a single stimulation of 1 Hz and 10 Hz LI-rTMS downregulated 21 genes at 5hrs post stimulation with no upregulated genes. There was only one gene that overlapped between 1 Hz and 10 Hz stimulation, emphasising frequency-specific effects for LI-rTMS of astrocytes Most of genes were related to inflammation, but there were also 6 signalling genes, 3 plasticity relate genes and 2 cytoskeletal-related genes (Clarke et al., 2021). Follow up protein analysis of Stim1, Orai3, Kcnmb4, and Ncam1 investigated whether gene changes were reflected in protein levels. Overall, there was significant reduction of STIM1, ORAI3, KCNMB4, and NCAM1 protein compared to sham after 10 Hz rMS, but significant increase in STIM1 and ORAI3 protein compared to sham after 1 Hz rMS (Clarke et al., 2021). Therefore LI-rTMS can alter expression of protein related to calcium signalling and cell adhesion. Functionally, these genetic and protein changes may influence anti-inflammatory and pro-survival effects as suggested in many of the studies of LI-rTMS in stroke, as well as general facilitation and inhibition of brain activity (Clarke et al., 2021).

Given the distinct mechanisms called into play by these two cell types, further studies using mixed cultures, *ex vivo* organotypic slices and organoid systems are necessary to investigate how LI-rTMS may influence neuron-glia interactions and bridge the gap between *in vitro* and *in vivo* experiments.

4. Magnetic-field effect

An important consideration in the PMF and rTMS literature is that magnetic fields themselves may play a role in biological changes, one that may be distinct from the effects of induced electrical activity. There are biological processes that are sensitive to magnetic fields and therefore may play a mechanistic role in PMF and rTMS effects in a way that makes magnetic field-based stimulation different to other brain stimulation tools such as transcranial electrical stimulation or optogenetics.

4.1. LI-rTMS and cryptochromes

Cryptochromes are proteins that are possible magnetoreceptors and are thought respond to the presence of a magnetic field through formation of radicals which induce changes in protein conformation (for review see Hore and Mouritsen, 2016). Some animals have the capacity to detect very low levels of magnetic fields - for example the Earth's magnetic field – which is 0.02–0.06 mT (Alken et al., 2021) – is thought to activate cryptochromes in the retinas of migratory birds to act as a magnetic compass (Wiltschko and Wiltschko, 2005). Therefore LI-rTMS levels are approximately 2–3 orders of magnitude greater than what is found naturally to activate these processes in rodent models.

For mammals, cryptochromes are best known in the brain for their role in regulating circadian rhythm, particularly in the suprachiasmatic nucleus. Activation of cryptochrome receptors via LI-rTMS has several possible effects. For example, activation of cryptochromes may increase cryptochrome related signalling which is involved in various pathways of metabolic and circadian control. A recent study applying 50 mT of 25 Hz magnetic stimulation looked at changes to circadian oscillation following stimulation of slices of rat suprachiasmatic nucleus in different phases of the circadian cycle (Kassahun et al., 2020). They found that stimulation at the lowest phase of the circadian cycle desynchronised and extended the circadian period by 2.47 h. Stimulation at the peak of the circadian cycle synchronised the circadian cycle and shortened the circadian period by 1.46 h. The study suggests that LI-rTMS is able to modulate circadian rhythm and that timing of stimulation also interacts with rTMS effects.

In the broader literature for circadian control and rTMS, sleep disturbances have been associated with treatment responses following HI-rTMS. In major depressive disorder greater sleep disturbances were associated with rTMS responders (Brakemeier et al., 2007), but in obsessive compulsive disorder, pronounced sleep disturbances were associated with rTMS non-responders (Donse et al., 2017). Therefore, further studies investigating the effect of both HI- and LI-rTMS on circadian oscillation, and the influence of stimulation during different phases of the circadian cycle could have significant impact, especially with growing evidence that cryptochromes are important for regulating both circadian and emotional responses (e.g. Griesauer et al., 2014). Cryptochromes could be an important mediator for various behavioural traits relevant to disorders treated with rTMS. For example, cryptochrome knockout mice have altered anxiety and depressive-like behaviour (De Bundel et al., 2013; Savalli et al., 2015; Schnell et al., 2015; Porcu et al., 2020; Sokolowska et al., 2021), impaired habituation (Hühne et al., 2020), and increased amygdala activity (Hühne et al., 2020). Furthermore, there is evidence that cryptochromes mediate repression of glucocorticoid receptors as the loss of cryptochrome genes is associated with high levels of circulating corticosterone (Lamia et al., 2011). The finding implicates cryptochromes in controlling suppression of the hypothalamic-pituitary-adrenal (HPA) axis, another important system in mood disorders. The mechanisms that may underpin these behaviours remain unknown although there is evidence that metabolism may be a mediating factor as cryptochrome knockout mice have significant changes to metabolic activity (Lamia et al., 2011; Barclay et al., 2013; De Bundel et al., 2013; Griebel et al., 2014; Savalli et al., 2015).

Recently, Sherrard et al. (2018) investigated the role of the cryptochrome in the effectiveness of PMF stimulation. Insect cells that

overexpressed DmCry, a Drosophila cryptochrome, had a significant increase in ROS concentration following PMF compared to unstimulated cells. Insect cells lacking DmCry, on the other hand, showed no effect of PMF on ROS accumulation. This pattern of cryptochrome-dependent increase in ROS concentration following PMF was replicated in murine and human cell lines with cryptochrome (Cry1/Cry2) knockout or knockdown. Furthermore, a microarray analysis of gene expression in HEK293 cells that did or did not receive 3 h of PMF revealed that a significant proportion of the regulated genes were associated with increased production of ROS and ROS-responsive genes. A follow-up study also demonstrated that cryptochrome is required for LI-rTMS-induced reinnervation following cerebellar lesions (Dufor et al., 2019). As described earlier, cerebellar LI-rTMS following a unilateral olivocerebellar lesion can induce olivocerebellar reinnervation both *in vivo* and *ex vivo* in wildtype mice (Morellini et al., 2015; Dufor et al., 2019). However, *ex vivo* explants from cryptochrome double knockout ($Cry1^{-/-}Cry2^{-/-}$) mice did not show LI-rTMS induced reinnervation, demonstrating that LI-rTMS induced reinnervation is dependent on the presence of cryptochrome (Dufor et al., 2019). Interestingly, in the cryptochrome knockout explants, external BDNF treatment was able to restore cerebellar reinnervation. Therefore it appears that the lack of a cryptochrome does not disrupt the ability of BDNF to induce reinnervation, but specifically blocks LI-rTMS plasticity, possibly by preventing the ability of rTMS to upregulate the BDNF pathway (Dufor et al., 2019). Dufor et al. (2019) also showed changes in expression of various genes associated with neurotrophic signalling after LI-rTMS. Several of these genes, also had binding sequences for CLOCK or ARNTL, which are complexes that drive the transcription of cryptochromes (Lin and Todo, 2005). These important experiments show that the magnetic field itself is integral to the regenerative effects of LI-rTMS and are also likely to be involved in HI-rTMS.

Based on the genetic changes described above, one possible mechanism for LI-rTMS effects is via genetic changes driven by cryptochrome activation. Altered protein conformation following cryptochrome activation may alter transcriptional control over circadian transcriptional complexes which regulate a wide range of genes (Lin and Todo, 2005). Furthermore, formation of radicals upon activation of cryptochromes may increase ROS expression and activate various ROS pathways (Ray et al., 2012; Arthaut et al., 2017). The wide spectrum of effects makes cryptochromes a compelling mechanism for the effects of electromagnetic stimulation.

4.2. LI-rTMS and the cell membrane

In addition to cryptochrome effects, the magnetic field may induce other biological changes independent of electric-field effects. For example, Goodman et al. (1986) used a model of cultured *Physarum polycephalum* amoebae (i.e. slime mold) to examine effects on the surface properties of the cell membrane. Amoebae received 25 Hz, 2 mT PMF (magnetic and electric stimulation) or an electric-field only stimulation, and surface charge and surface lipid and protein content (hydrophobicity) were assessed. Both the PMF and electric field only stimulation made the surface charge more negative, but only the PMF reduced cell surface hydrophobicity by altering the lipid and protein content of the cell membrane.

Extending these findings into eukaryotic cells, SH-SY5Y neuronal-like cells were stimulated with either 1 mT 50 Hz PMF or static magnetic stimulation and their plasma membranes were analysed using Fourier Transform Infrared Spectroscopy (FTIR) (Calabrò and Magazù, 2019). FTIR is an infrared scanning technique that can be used to study the molecular composition and dynamics of biological samples (Baker et al., 2014). The FTIR analysis showed that exposure to both static and pulsed magnetic fields at 1 mT altered the orientation of membrane protein α-helices to align with the direction of the magnetic field (Calabrò and Magazù, 2019). Because α-helices are commonly found in transmembrane receptors including ion channels, a possible outcome would be an altered channel pore size, resulting in changes to the flux of ions across the membrane and thus to membrane potential. In support of the possibility, mitochondrial transmembrane potential decreased after PMF (Calabrò et al., 2013b) and static magnetic field (Calabrò et al., 2013a) exposure. In addition to changing protein alignment, FITR suggested that static and pulsed magnetic fields may also cause realignment of lipid chains, increasing the cell membrane surface area (Calabrò and Magazù, 2019). The finding matches with evidence of changes to hydrophobicity, a measure of surface lipid content, following PMF (Goodman et al., 1986). However direct morphological analysis has not yet been completed, so changes remain theoretical.

Overall, there is compelling evidence that the magnetic field component of LI-rTMS and PMF may exert relevant biological effects through direct interactions with cryptochrome proteins, as well as by biomagnetic effects on important protein and lipid molecules within the cell. It will be important to investigate these mechanisms further for their potential to deliver therapeutic effects independently of the induced electric field.

5. Summary

Low-intensity brain stimulation has been demonstrated to be a versatile tool across a variety of domains. Research over the past decade has shown LI-rTMS changes to brain excitability, metabolism, neurotransmitter release, and connectivity, improvements in learning and symptoms of tinnitus and mood disorders, reorganisation of abnormal circuits through the upregulation of various factors, as well as neuroprotective and regenerative effects after brain injuries. New research is elucidating several of the possible mechanisms behind these changes, reporting a range of pathways through which LI-rTMS influences the brain. LI-rTMS appears to improve and maintain cell survival, modulate various growth and nerve factors such as BDNF which promote regeneration and plasticity in neurons and modulate cellular activation, even at sub-action potential threshold intensities. A major mechanism appears to be changes to calcium-signalling induced by LI-rTMS which are maintained even after stimulation stops. Changes to glia functioning are also linked to these calcium changes, with LI-rTMS related changes shown across microglia, astrocytes, and oligodendrocytes. Changes to these glial cell types have modulatory and network level implications that we are only now beginning to unravel, both for injured and healthy brains. Finally, LI-rTMS has also been linked with transcriptional changes which suggest impact across a breadth of cellular and network effects.

Here we flag areas of particular interest:

1. More research into mechanisms

Despite the significant amount of rTMS research, our review has highlighted key areas that will provide benefits: by highlighting new mechanisms, we can identify new disorders that may benefit from LI-rTMS.

Multicellular effects of LI-rTMS: it will be interesting to elucidate how LI-rTMS influences the interactions between glia and neurons, particularly in relation to calcium changes. Research on oligodendrocyte plasticity suggests that glia have a key role in mediating LI-rTMS effects and this research has already been translated to a clinical trial for multiple sclerosis (Australian New Zealand Clinical Trials Registry, Registration number: ACTRN12619001196134).

Metabolic changes: despite extensive indirect/suggestive evidence for metabolic changes after LI-rTMS there has been very little research into modulation of metabolic pathways, and based on existing evidence, experiments should consider the potential for mitochondrial changes.

Behaviour: Further research to link behavioural and molecular changes would provide a better understanding of how behavioural changes can be maintained after stimulation stops, a key feature in developing effective treatments for patients. The small size of LI-rTMS coils allows flexibility in experimental design as LI-rTMS can be

delivered online or offline (Poh et al., 2018; Moretti et al., 2021), to explore timing of stimulation (priming, concurrent or consolidatory stimulation).

Timing of mechanism: The chronological and causal sequence of molecular and cellular events immediately following LI-rTMS is not fully characterised. For example, although it is clear that LI-rTMS can induce an increase in calcium levels in neurons and astrocytes within minutes after the start of stimulation (Grehl et al., 2015; Clarke et al., 2017b; Ye et al., 2020), it is not clear whether this is a cause or a consequence of increased neural activity. Stimulation may increase neural activity, resulting in calcium release, or stimulation may directly induce calcium release which then influences neural activity. Both processes may also occur at the same time. Importantly, the specific downstream targets of LI-rTMS-induced calcium change have yet to be elucidated. It will be important in future to dissect out the timing and relative contributions of these important pathways to LI-rTMS facilitation of reinnervation, regeneration and synaptic plasticity. For example, making use of synaptic live cell imaging and *in vivo* imaging technology in experiments could begin to help characterise early stages of rTMS to delve deeper into what processes are key for triggering rTMS-induced plasticity.

2. Clinical implications

Translation: The preclinical research base for LI-rTMS is relevant to disorders such as tinnitus, depression and stroke, providing huge translational potential. In addition, there are currently human LI-rTMS trials underway which should release results in the next 12–24 months for depression and multiple sclerosis. The results of these initial studies could influence future guidelines and clinical practice and give rise to further trials.

Relevance: Preclinical LI-rTMS research is relevant to understanding rTMS as it is currently used in the clinic. As discussed in section 1, during HI-rTMS, peripheral low-intensity fields may be contributing to rTMS effects, and studying LI-rTMS offers insight into these peripheral effects. For animal models of rTMS, the smaller LI-rTMS coils allow focal stimulation rather than whole brain stimulation that occurs when current HI-rTMS coils are used in animals due to the relative size difference in animal vs. human heads and this could better model the stimulation set-up used for humans. There is also evidence that some therapeutic benefits or biomarkers may be specific to a particular intensity (Heath et al., 2018). Combined EEG-TMS or fMRI-TMS studies may be useful for investigating the role of lower intensities for changes in connectivity.

The biological effects of LI-rTMS also raise concerns over the design of control groups in research and clinical trials. HI-rTMS studies often use an 'active sham' condition that induces a weak electric field (for example using purpose designed sham coils, or repositioned standard coils) and may thus have confounding LI-rTMS effects (Loo et al., 2000; Harvey et al., 2018; Boucher et al., 2021). One example is the NICHE trial for hemiparesis which used a sham treatment that delivered stimulation in range of LI-rTMS. The trial demonstrated no significant difference between sham and active stimulation, but showed improvement in both conditions relative to rehabilitation without neuromodulation. Such 'active sham' conditions may be better interpreted as partially active shams since LI-rTMS may contribute its own effects. The other approach to sham controls is to use inactive stimulation (coil switched off or replica coil), but this does not control for scalp sensations and auditory stimuli, which may contribute to experimental outcomes through placebo or other mechanisms. Unfortunately there appears to be no "ideal" sham condition for rTMS.

Innovation: If LI-rTMS does prove effective in humans it would allow expansion of rTMS therapeutics. LI-rTMS is more tolerable than HI-rTMS, with no reported side-effects or any associated sensation. The reduced power requirements also simplify the engineering constraints. Lightweight, portable stimulators could be designed, similar to PMF stimulators, which could reduce cost and increase availability. Individuals may also be able to apply their own stimulation at home, under the direction of clinicians new pre-programmable transcranial electrical stimulation machines currently on the market, which would reduce the burden of lengthy, daily clinic visits for patients.

5.1. Conclusion

There is still a long way to go in LI-rTMS research, but there has been a level of breadth to the current research that encompasses a variety of models and mechanisms in a way that has not been possible with high intensity (clinical) rTMS due to the lack of suitable tools. Mechanisms at genetic, molecular, cellular and system levels have been identified with evidence that LI-rTMS and potentially rTMS in general acts through several key pathways to induce changes in the brain. Future research expanding LI-rTMS into human research while continuing to elucidate mechanisms in preclinical models holds promise for the future of neuromodulation.

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CRediT authorship contribution statement

Jessica Moretti: Conceptualization, Writing – original draft, Writing – review & editing. **Jennifer Rodger:** Conceptualization, Writing – review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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