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# Frequency-specific effects of low-intensity rTMS can persist for up to 2 weeks post-stimulation: A longitudinal rs-fMRI/MRS study in rats

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ABSTRACT

*Background:* Evidence suggests that repetitive transcranial magnetic stimulation (rTMS), a non-invasive neuromodulation technique, alters resting brain activity. Despite anecdotal evidence that rTMS effects wear off, there are no reports of longitudinal studies, even in humans, mapping the therapeutic duration of rTMS effects.
*Objective:* Here, we investigated the longitudinal effects of repeated low-intensity rTMS (LI-rTMS) on healthy rodent resting-state networks (RSNs) using resting-state functional MRI (rs-fMRI) and on sensorimotor cortical neurometabolite levels using proton magnetic resonance spectroscopy (MRS).
*Methods:* Sprague-Dawley rats received 10 min LI-rTMS daily for 15 days (10 Hz or 1 Hz stimulation, n = 9 per group). MRI data were acquired at baseline, after seven days and after 14 days of daily stimulation and at two more timepoints up to three weeks post-cessation of daily stimulation.
*Results:* 10 Hz stimulation increased RSN connectivity and GABA, glutamine, and glutamate levels. 1 Hz stimulation had opposite but subtler effects, resulting in decreased RSN connectivity and glutamine levels. The induced changes decreased to baseline levels within seven days following stimulation cessation in the 10 Hz group but were sustained for at least 14 days in the 1 Hz group.
*Conclusion:* Overall, our study provides evidence of long-term frequency-specific effects of LI-rTMS. Additionally, the transient connectivity changes following 10 Hz stimulation suggest that current treatment protocols involving this frequency may require ongoing "top-up" stimulation sessions to maintain therapeutic effects.



## Introduction

Repetitive transcranial magnetic stimulation (rTMS) is a non-invasive neuromodulation technique widely applied in therapeutic and investigative studies of neuropsychiatric conditions including depression [1,2], schizophrenia [3], and Parkinson's disease [4]. Despite increasing use of rTMS to modulate dysfunctional brain networks in humans, the mechanisms underlying its therapeutic effects, particularly in cortical circuits, still require elucidation. Furthermore, patients report that therapeutic benefits of rTMS wear off after treatment finishes [e.g., Refs. [5,6]], suggesting an urgent need to characterise the persistence of rTMS effects.

Therapeutic rTMS application utilises different stimulation frequencies to elicit different cortical changes: low-frequency (<5 Hz) stimulation has inhibitory and high-frequency (>5 Hz) has excitatory effects on the cortex, albeit with some individual variability [7,8]. Preclinical models have identified gene and protein expression changes associated with these frequency-specific changes in excitatory and inhibitory circuit activity [9–12]. In addition, changes in neurotransmitters glutamate (Glu), γ-aminobutyric acid

*Abbreviations:* C1, interoceptive/DMN network; C2, cortico-striatal-thalamic network; C3, basal ganglial network; C4, salience network; SD7, after seven days of daily stimulation; SD14, after 14 days of daily stimulation; PSD7, seven days post-stimulation cessation; PSD14, 14 days post-stimulation cessation; and PSD20, 20 days post-stimulation cessation.

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(GABA), and glutamine (Gln), the major precursor for neuronal glutamate and GABA [13], are central to excitability changes observed following stimulation [9,14,15]. High-frequency stimulation increases excitatory neuronal activity [16,17] and induces a long-lasting increase in glutamatergic synaptic strength consistent with long-term potentiation (LTP) of excitatory circuits [9], while simultaneously reducing the strength of GABAergic synapses [11,18]. Conversely, 1 Hz stimulation increases inhibitory circuit activity by increasing GABA levels [19] and modulates expression of calcium-binding proteins in inhibitory interneurons [10,12].

Although animal models have revealed some key mechanisms of rTMS, in most preclinical studies, changes in the brain are investigated post-mortem, providing only a snapshot of rTMS-induced changes. Additionally, most animal and human studies focus on events occurring minutes to hours after single or multiple stimulations. Consequently, the development of rTMS effects over time, and the stability of its after-effects, have not been explored.

Non-invasive magnetic resonance (MR) based techniques are ideal for longitudinal studies of brain function and neuroplasticity [20]. Resting-state functional magnetic resonance imaging (rs-fMRI) is the technique of choice for examining long-term changes in functional networks [21–25], while proton ($^1$H) magnetic resonance spectroscopy (MRS) is one of only few methods that can non-invasively assay neurometabolic changes. Rs-fMRI identifies brain regions showing synchronised resting activity which form organised networks called resting-state networks (RSNs) [26]. RSN dysregulations, and accompanying neurometabolic changes, have been identified in patients with neuropsychiatric disorders [27], e.g., schizophrenia and depression [28]. Both RSN functional connectivity (FC) and neurometabolite levels in humans are sensitive to rTMS and clinical improvements are associated with changes in RSNs [for review, see 25]. However, there have been no reports of longitudinal studies to characterise the timecourse of changes in RSNs or neurometabolites during and after rTMS treatment.

A single session of low-intensity rTMS (LI-rTMS) in rats has frequency-specific effects on RSNs similar to those described in humans following rTMS [21]. This evidence of translational validity suggests that LI-rTMS in rodents can be a useful model in a translational pipeline to inform and guide clinical application of rTMS, particularly given the cost and logistical challenges of longitudinal human studies. For example, recent active fMRI/rTMS studies in animal models of traumatic brain injury have shown that repeated delivery of high-frequency rTMS increased primary somatosensory activity in response to forelimb stimulation, indicating restored cortical function [29,30]. The present study used rs-fMRI and MRS in rats to investigate the emergence of LI-rTMS-induced changes in FC and neurometabolite levels respectively and their maintenance for up to three weeks. Two weeks of daily stimulation resulted in significant changes in FC and neurometabolites that outlasted the duration of stimulation. A better understanding of rTMS effects on RSNs and neurometabolites may prove helpful in the development of long-lasting treatment options to modify dysfunctional connectivity detected in several neuropsychiatric disorders.

## Materials and methods

### *Animals*

Experimental procedures were approved by the UWA Animal Ethics Committee (RA/3/100/1430) in accordance with the *Australian code for the care and use of animals for scientific purposes*. 18 adult male Sprague-Dawley rats (6–8 weeks old, 150–250 g) from the Animal Resources Centre (Canning Vale, Western Australia) were maintained in a temperature-controlled animal care facility on a 12-h light-dark cycle with food and water *ad libitum*. Animals were euthanised after the last imaging session using $CO_2$ asphyxiation.

### *Experimental protocol*

Following acquisition of baseline rs-fMRI data, all animals received daily 10 min LI-rTMS at 10 Hz or 1 Hz for 15 days (n = 9/group, Fig. 1). LI-rTMS was delivered using a custom-built round coil [described in detail in Refs. [21,31]] held by the experimenter between right eye and ear (~13 mT at cortical surface). Animals were habituated to handling and to the coil for one week, as described previously [11,32]. Imaging was conducted under isoflurane-medetomidine combination anaesthesia weekly during stimulation. Another two imaging sessions were performed seven and 20 days after stimulation was ceased for the 10 Hz group, and seven and 14 days after stimulation was ceased for the 1 Hz group. The only difference between the imaging timeline of the two groups was the timing of the last imaging session (20 days after stimulation cessation for the 10 Hz group and 14 days after stimulation cessation in the 1 Hz group) due to MRI hardware failure that delayed scanning of the 10 Hz group. After the scanning procedure, medetomidine was antagonised by an injection of atipamezole. See supplementary methods for further details, drug dosing, etc.

### *Data acquisition*

All MR images were acquired with a 9.4 T Bruker Biospec 94/30 small animal MRI using the imaging protocol as described in the supplementary methods and in Ref. [21]. Briefly, the acquisition protocol included the following sequences: 1) multi-slice 2D RARE (rapid acquisition with relaxation enhancement) sequence for three T2-weighted scans (TR = 2500 ms, TE = 33 ms, matrix = 280 × 280, 21 coronal and axial slices, 20 sagittal slices, thickness = 1 mm); 2) single-shot gradient echo EPI (TR = 1500 ms, TE = 11 ms, matrix = 94 × 70, 21 coronal slices, thickness = 1 mm, pixel size = 0.3 × 0.3 mm$^2$, flip angle = 90°, 300 vol) for resting-state; and 3) point-resolved spectroscopy (PRESS) sequence with one 90° and two 180° pulses, and water suppression for $^1$H-MRS (TE = 16 ms, TR = 2500 ms) with 64 averages (Table A.1) with a 3.5 × 2 × 6 mm$^3$ voxel placed over the right sensorimotor cortex (Fig. 2).

### *Image processing and analysis*

Image processing was performed as described in Seewoo et al. [21], with the following changes: 1) qimask utility from QUIT (QUantitative Imaging Tools) used for skull-stripping [33]; 2) Gaussian kernel filter was set to a full-width half maximum of 6.25 mm for single-session independent component analysis (ICA); and 3) FIX (FMRIB's ICA-based Xnoiseifier v1.06; threshold 20) was trained to automatically remove noise components (See Supplementary Methods for more details) [34,35].

Because of the exploratory nature of this study, an ICA-based approach was used to identify whole-brain FC changes. Multi-subject temporal concatenation group-ICA was carried out on baseline rs-fMRI data using MELODIC to identify template RSNs (Fig. 3). The ICA algorithm was restricted to 15 components based on other rs-fMRI studies in rodents [21,36,37]. Dual regression analysis was then conducted on relevant RSNs for between-timepoint comparisons [38,39], controlling for family-wise error (FWE) and using a threshold-free cluster enhanced (TFCE) technique to control for multiple comparisons ($P < 0.05$). Regions showing significant differences (Figs. 4 and 5) were then labelled using a rat brain atlas [40].

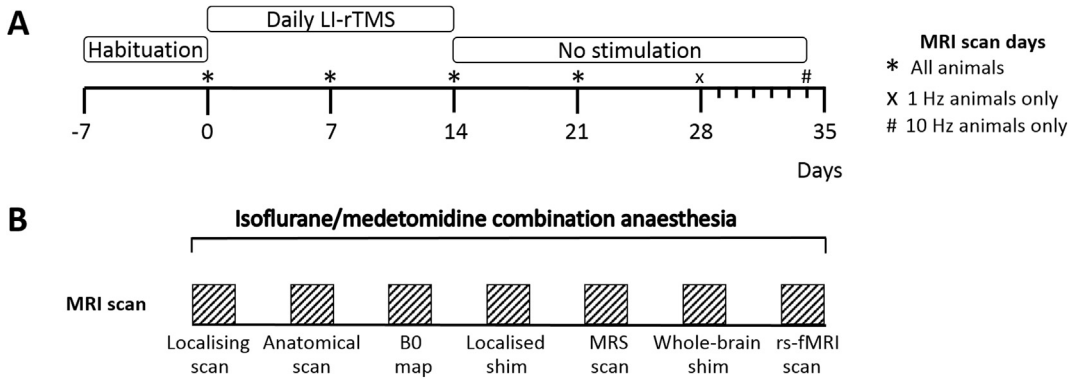


**Fig. 1. Timeline of an experiment for one rat.** A. The experiment consisted of an initial one-week period of habituation upon arrival of the animal, after which the rats had five sessions of MRI scans, each separated by at least one week. Day 0 was the first imaging session for acquiring baseline resting-state activity and neurometabolite levels. For the first two weeks, animals were stimulated daily for 10 min at 10 Hz (6000 pulses) or 1 Hz (600 pulses). Stimulation was ceased after 15 days of stimulation (after Day 14), but the animals were still imaged on Day 21 (all animals), Day 28 (1 Hz animals) and Day 34 (10 Hz animals). These five imaging timepoints will be referred to as: baseline, after seven (SD7) and 14 days (SD14) of daily stimulation and either 20 days (10 Hz group) or 14 days (1 Hz group) after stimulation cessation (PSD20 or PSD14 respectively). B. Protocol for a single scan session. During each session, the animal was under a combination of isoflurane and medetomidine anaesthesia throughout the experiment. Each session consisted of the acquisition of a localising scan, an anatomical scan, a B0 map, a localised shim, an MRS scan, whole brain shim, and an rs-fMRI scan.

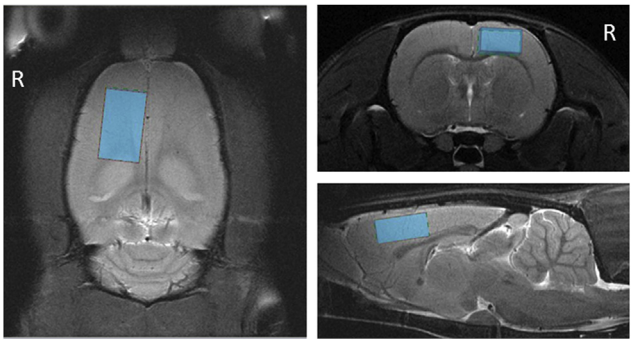


**Fig. 2. Volume of interest (VOI) positioning.** The figure shows the position of the MRS voxel (size of 3.5 mm × 2 mm × 6 mm) on the right sensorimotor cortex (ipsilateral to LI-rTMS delivery) on T2-weighted images for proton nuclear magnetic resonance spectroscopy.

*MRS data analysis and presentation*

MRS data was analysed in LCModel ("Linear Combination of Model spectra" version 6.3-1L) [41] using a simulated basis set provided by the software vendor. Individual metabolite concentrations were computed using the unsuppressed reference water signal for each individual scan. The Cramér-Rao lower bounds (CRLBs) were calculated by LCModel and reported as the percentage of the estimated concentrations to determine reliability of the metabolite estimates (see Table A.2 for quantification details). The metabolites of interest were GABA and Glu, the major neurotransmitters in the brain, Gln, a neurotransmitter precursor, and combined glutamate-glutamine (Glx). All results presented for the metabolite concentrations are expressed as Tau ratios (metabolite concentration/Tau concentration) because (i) Tau peak could be identified with high reliability by LCModel (CRLB < 10%), and (ii) to

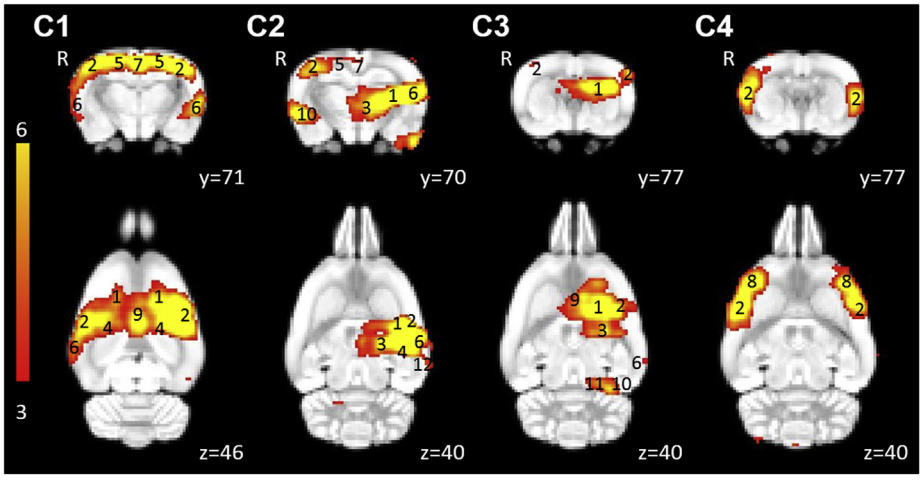


**Fig. 3. Template resting-state networks from baseline rs-fMRI scans.** The figure illustrates coronal and corresponding axial slices of four RSNs (C1–C4) that were identified in the baseline rs-fMRI scans of 6-7-week-old male Sprague Dawley rats under isoflurane-medetomidine combination anaesthesia. The four components were classified as follows: C1, interoceptive/DMN network; C2, cortico-striatal-thalamic network; C3, basal ganglial network; and C4, salience network. The spatial colour-coded z-maps of these components are overlaid on the rat brain atlas (down-sampled by a factor of eight) and the numbers on the bottom right corner of each slice refer to the position on the atlas. The RSN maps are represented as z-scores (n = 22, thresholded at z > 3), with a higher z-score (yellow) representing a greater correlation between the time course of that voxel and the mean time course of the component. R denotes right hemisphere. Significant clusters within the components include various brain regions: 1, striatum/caudate putamen; 2, somatosensory cortex; 3, thalamus; 4, hippocampus; 5, motor cortex; 6, auditory cortex; 7, retrosplenial cortex; 8, insular cortex; 9, cingulate cortex; 10, entorhinal cortex; 11, inferior colliculus; 12, association cortex. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

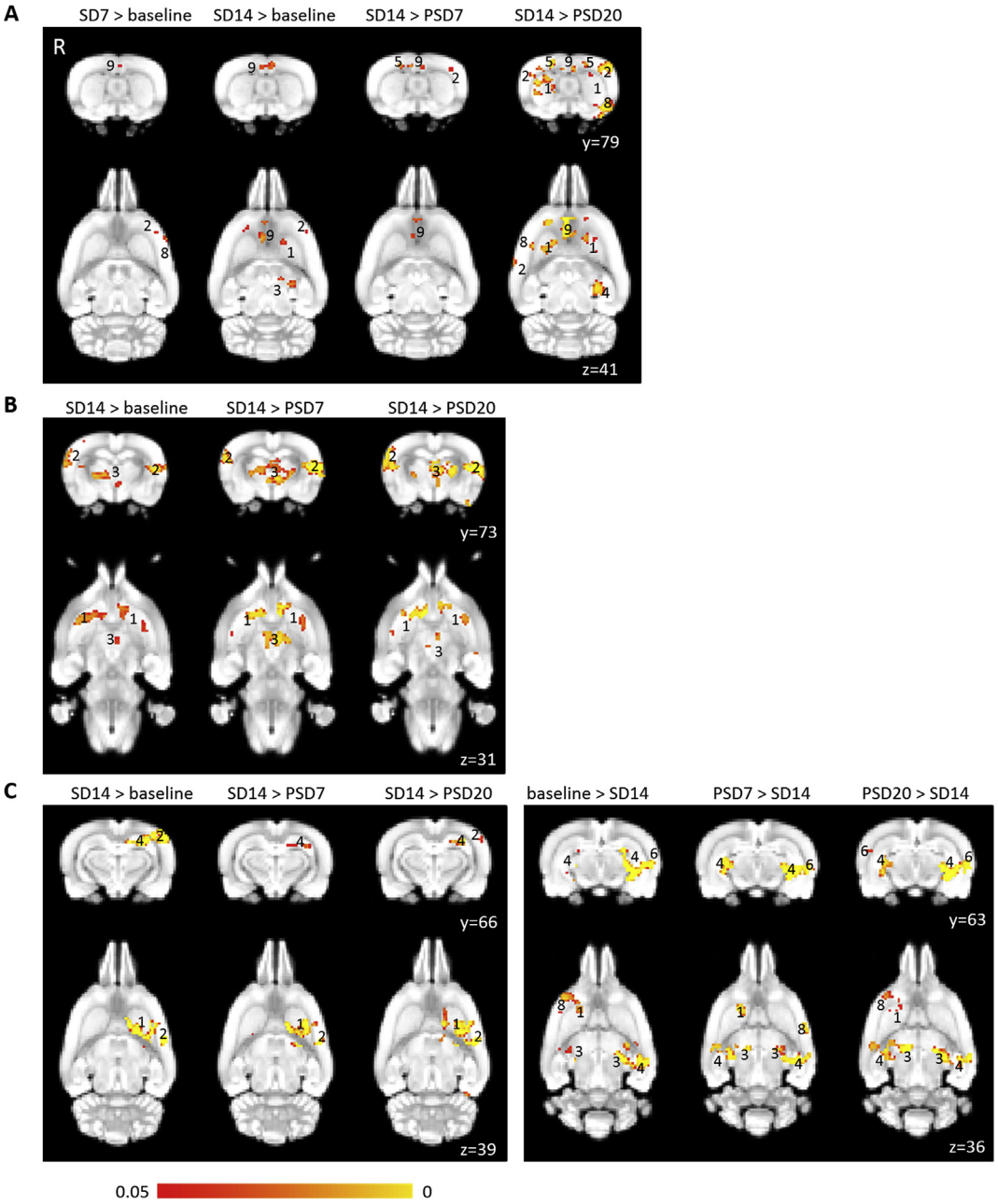


**Fig. 4. Functional connectivity changes within the (A) interoceptive/default mode network, (B) cortico-striatal-thalamic network and (C) basal ganglial network induced by 10 Hz LI-rTMS.** The figure illustrates changes between five timepoints: baseline, after seven (SD7) and 14 days (SD14) of daily stimulation and after seven (PSD7) and 20 days (PSD20) post-stimulation cessation. The spatial colour-coded p-value maps of these components are overlaid on the rat brain atlas (down-sampled by a factor of eight) and the numbers on the right refer to the slice position on the atlas. The dual regression maps are represented as p-values corrected for multiple comparisons at voxel level ($n = 9$, thresholded at $P < 0.05$). R denotes right hemisphere. Significant differences were found in: 1, striatum/caudate putamen; 2, somatosensory cortex; 3, thalamus; 4, hippocampus; 5, motor cortex; 6, auditory cortex; 8, insular cortex; 9, cingulate cortex. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

the best of our knowledge, there is no literature showing high (HI) or low intensity rTMS-related changes in Tau. To improve sensitivity for detecting changes in glutamate-glutamine cycle function, we also report results for Glu/Gln ratios for detecting LI-rTMS-induced changes in neurotransmission. Analyses and plots of metabolite ratios were carried out using RStudio version 3.5.2 [42]. Three data points from each group were excluded from the analyses due to identification as outliers by boxplots, bad shimming and/or bad voxel positioning leading to high CRLB values. Repeated-measures ANOVA ('lme4' and 'lmerTest' packages) was utilised to test for between-timepoint differences. When significant main effects of timepoints were found, *post hoc* pairwise differences were calculated ('glht' in 'multcomp' package) to determine significant changes in metabolite ratios between two timepoints (Fig. 6). Tukey all-pair comparisons were carried out and Bonferroni-Holm adjusted p values reported.

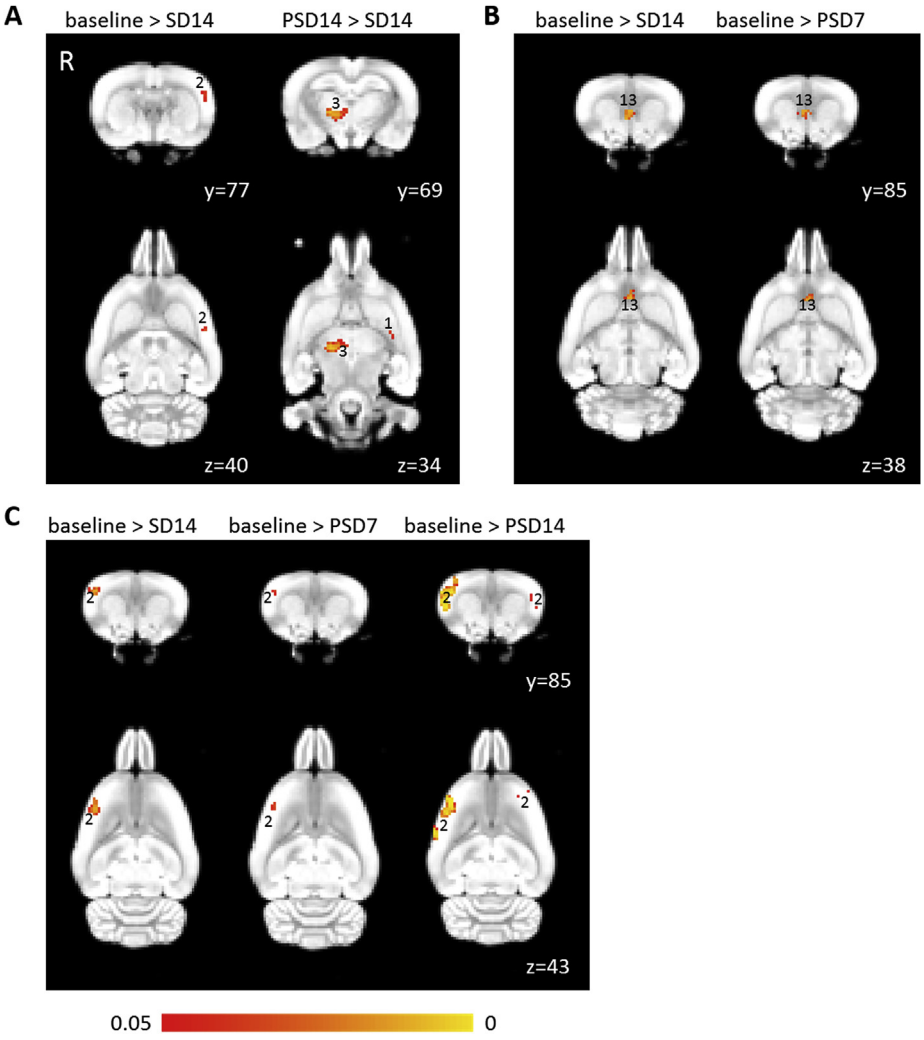


**Fig. 5. Functional connectivity changes within the (A) cortico-striatal-thalamic network, (B) basal ganglial network and (C) the salience network induced by 1 Hz LI-rTMS.** The figure illustrates changes between five timepoints: baseline, after seven (SD7) and 14 days (SD14) of daily stimulation and after seven (PSD7) and 14 days (PSD14) post-stimulation cessation. The spatial colour-coded p-value maps of these components are overlaid on the rat brain atlas (down-sampled by a factor of eight) and the numbers on the right refer to the slice position on the atlas. The dual regression maps are represented as p-values corrected for multiple comparisons at voxel level ($n = 9$, thresholded at $P < 0.05$). R denotes right hemisphere. Significant differences were found in: 1, striatum/caudate putamen; 2, somatosensory cortex; 3, thalamus; 13, prelimbic cortex. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

## Results

### *Template RSNs identified by group-ICA*

Template rodent RSNs were identified from baseline data to avoid including LI-rTMS-related RSN alterations in the template components and thus increase the sensitivity of dual regression in detecting between-group differences [39]. Based on visual inspection of the 15 components identified by group-ICA, four components (Fig. 3) were identified as classical rodent RSNs [e.g., Refs. [37,43–45]]. The remaining eleven components were classified as noise (see examples in Fig. A1). Component 1 is dominated by a cortical ribbon, and interoceptive [44] and default mode network (DMN) [46] structures (Table 1) are grouped to form a network. Component 2 (cortico-striatal-thalamic network) includes similar structures as C1 (see Table 1 for differences). A similar network was identified by Ref. [43] in infant rats, without the high correlation in the cortex seen in C2 but with a greater correlation to the hippocampus. Component 3 (basal ganglial network) mostly involves subcortical regions (Table 1), similar to the network reported in infant rats by Bajic et al. [43]. Component 4 presents characteristics of the salience network, showing specific overlap with the insular and somatosensory cortices [43].

### *Functional connectivity changes*

Rs-fMRI data was acquired at five timepoints: baseline, after seven stimulation sessions (SD7), after 14 stimulation sessions (SD14), seven days after stimulation cessation (PSD7), and either 20 days (10 Hz group) or 14 days (1 Hz group) after stimulation cessation (PSD20 or PSD14 respectively). Overall, dual regression revealed that 10 Hz LI-rTMS induced significant potentiation of FC in C1, C2, and C3 (Fig. 4) while 1 Hz LI-rTMS significantly attenuated FC in C2, C3, and C4 (Fig. 5).

Daily 10 Hz stimulation significantly increased FC of several brain regions to C1 by SD7 (Fig. 4A) and this increase was even greater on SD14. Following stimulation cessation, C1 FC decreased back to baseline levels over 20 days, with the drop being more significant and widespread on PSD20 than on PSD7. There were no significant differences between PSD7 and PSD20 or between

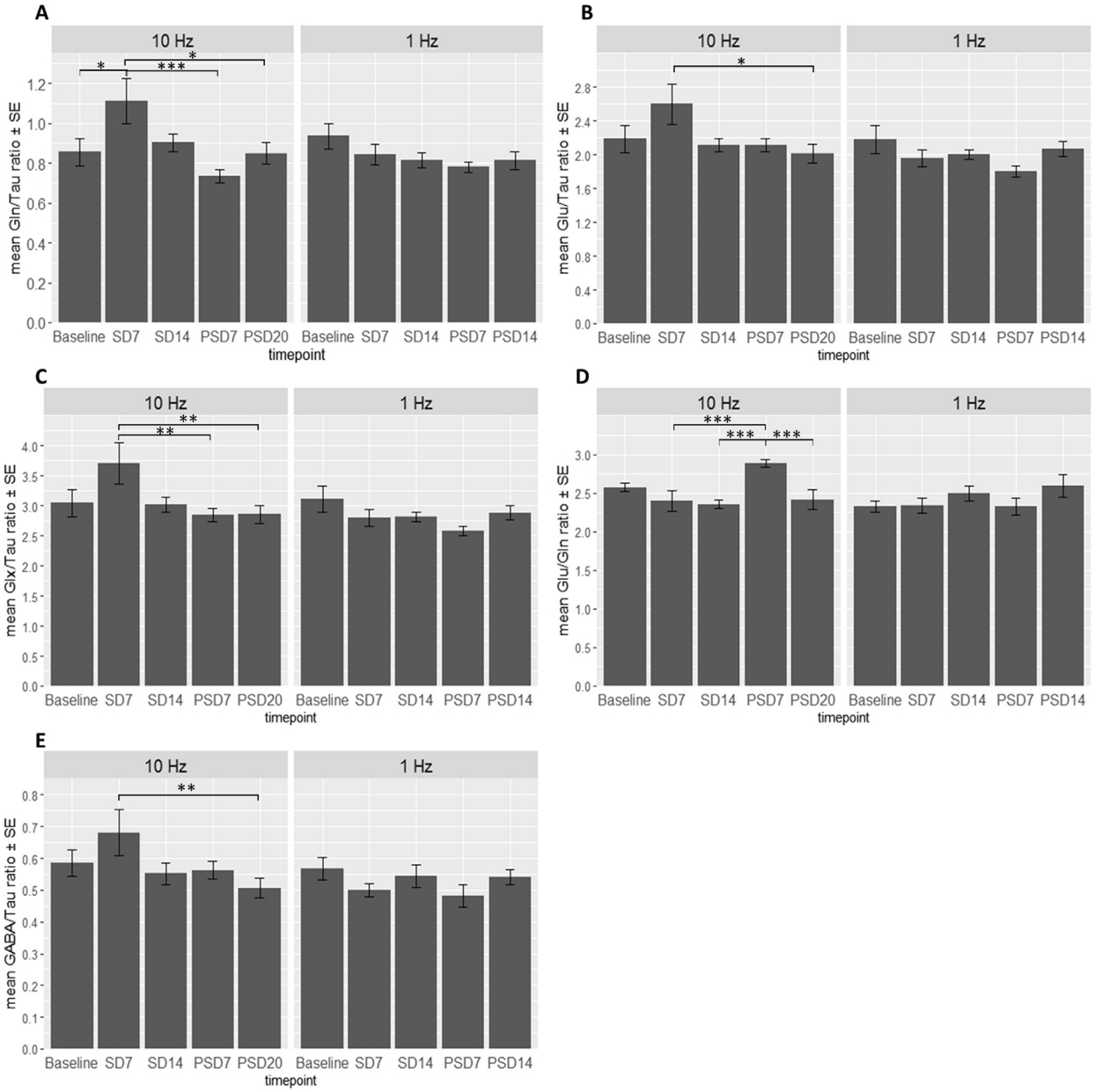


**Fig. 6. Longitudinal changes in metabolite ratios at the different timepoints in rat sensorimotor cortex following 10 Hz and 1 Hz LI-rTMS.** All graphs show mean ± standard error and *post hoc* pairwise differences results (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). The five metabolite ratios studied were as follows: A, glutamine (Gln)/Tau ratio; B, glutamate (Glu)/Tau ratio; C, glutamine + glutamate (Glx)/Tau ratio; D, glutamate/glutamine (Glu/Gln) ratio; and E, γ-aminobutyric acid (GABA)/Tau ratio.

**Table 1**
Summary of brain regions within the four components identified by group-ICA.

| Components | Networks | Brain regions |
|---|---|---|
| **C1** | Interoceptive/default mode network | Cortical regions, e.g., the somatosensory cortex, motor cortex, auditory cortex, retrosplenial cortex, and cingulate cortex as well as the striatum and hippocampus. |
| **C2** | Cortico-striatal-thalamic network | Similar to C1, excluding the cingulate cortex but including the entorhinal cortex. Predominantly within the cortex, striatum, and thalamus. |
| **C3** | Basal ganglial network | Subcortical, mostly the striatum. |
| **C4** | Salience network | Insular and somatosensory cortex. |

baseline and both PSD7 and PSD20, showing that FC returned to baseline within one week after stimulation was stopped. C2 FC was unchanged on SD7 but significantly increased by SD14 (Fig. 4B). FC returned to and remained stable at baseline by PSD7. Similarly, C3 FC to the left somatosensory cortex, striatum and dorsal hippocampus peaked at SD14 (Fig. 4C) and decreased back to baseline by PSD7. Surprisingly, C3 FC on SD14 was also found to be significantly reduced in the right striatum, insular cortex, bilateral ventral hippocampus, thalamus, and auditory cortex, returning to baseline by PSD7 as well (see more detailed changes in Table A.3).

In the 1 Hz group, C2 FC to the left somatosensory significantly dropped after 14 stimulation sessions (Fig. 5A). C2 FC to the right thalamus and left striatum was elevated on PSD14 compared to SD14 despite there being no significant changes in these regions compared to baseline at both timepoints. Additionally, there was a significant decrease in C3 FC on SD14 (Fig. 5B) that was sustained on PSD7 but had returned to baseline by PSD14. In contrast, the decreased C4 FC to the right somatosensory cortex noted on SD14 stayed below baseline on PSD7 and had further decreased by PSD14 to also encompass the left somatosensory cortex (Fig. 5C).

*Changes in metabolite ratios*

To gain further insight into the association between FC and neurometabolic changes, we measured Gln, Glu, Glx and GABA levels in the sensorimotor cortex using $^{1}$H-MRS. Please note that herein we have chosen to report the MRS data as metabolite ratios relative to Tau, for the following reasons. Typically, *in-vivo* $^{1}$H-MRS studies report metabolite concentrations in a relative manner using an internal concentration reference: water or any specific metabolite [47,48]. The use of water as reference signal requires several uncertain corrections, for example due to partial volume and relaxation effects [49]. Moreover, the water concentration in brain tissue has to be determined because water signal arises from a combination of brain tissue and cerebrospinal fluid while detectable metabolites are exclusively found in brain tissue [50,51]. Metabolite concentration ratios, as reported in this study, are less sensitive to these effects. The choice of the metabolite reference is arbitrary unless the metabolite concentration is expected to vary between groups and timepoints [48]. To our knowledge, there is no literature showing rTMS-related changes in Tau, and accordingly we have chosen Tau as a concentration reference in our study (see Table A.5 for water-referenced metabolite concentrations in institutional units).

Spectra obtained were reproducible longitudinally (see example in Fig. A2). Unpaired Welch Two Sample *t*-test showed no significant differences in the baseline metabolite/Tau ratios between 10 Hz and 1 Hz groups.

Following 10 Hz stimulation, repeated-measures ANOVA revealed a main effect of timepoint for all five metabolite ratios investigated (Table 2). *Post hoc* comparisons showed a significant increase in Gln on SD7 compared to baseline (Fig. 6), while Glu, Glx and GABA levels only increased non-significantly (see Table A.4 for mean metabolite concentrations). The levels of all four metabolites returned to baseline after an additional seven days of stimulation (i.e., no significant difference between baseline and SD14). After cessation of stimulation, Gln and Glx levels on PSD7 and PSD20 were significantly lower than on SD7. In contrast, Glu and GABA levels were only significantly lower than SD7 on PSD20. On the other hand, Glu/Gln levels decreased non-significantly from baseline with daily stimulation and increased significantly between SD7 and PSD7, and between SD14 and PSD7. There was a significant drop in Glu/Gln level on PSD20 compared to PSD7, i.e., Glu/Gln level decreased back to baseline by PSD20.

**Table 2**
Summary of results from repeated-measures ANOVA of neurometabolite levels to test for main effect of timepoint.

| Group | Metabolite | *F* | *P* |
|---|---|---|---|
| **10 Hz** | **Gln/Tau** | 5.72 | 0.0016 ** |
| | **Glu/Tau** | 3.06 | 0.032 * |
| | **Glx/Tau** | 3.87 | 0.012 * |
| | **GABA/Tau** | 3.03 | 0.033 * |
| | **Glu/Gln** | 6.02 | 0.0012 ** |
| **1 Hz** | **Gln/Tau** | 2.35 | 0.078† |
| | **Glu/Tau** | 2.30 | 0.082† |
| | **Glx/Tau** | 2.56 | 0.059† |
| | **GABA/Tau** | 1.33 | 0.28 |
| | **Glu/Gln** | 1.48 | 0.23 |

Significance codes: $^{**}P < 0.01$, $^{*}P < 0.05$, $\dagger P < 0.1$.

For the 1 Hz group, repeated-measures ANOVA did not detect significant timepoint-related changes in any metabolite ($P < 0.1$ for Gln, Glu and Glx; and $P > 0.1$ for GABA and Glu/Gln). Gln ($P = 0.0873$), Glu ($P = 0.0444$) and Glx ($P = 0.0229$) levels decreased to lower than baseline on PSD7, after cessation of stimulation (Fig. 6).

## Discussion

rTMS therapy is normally delivered to patients as a course of treatment over several weeks to induce lasting plastic changes. However, the stability of these changes remains unknown. This study examined both the emergence and maintenance of changes in FC and neurometabolite levels, assessed with rs-fMRI and MRS respectively, following repeated LI-rTMS in rats. Our study confirms the frequency-specific effects of LI-rTMS and further suggests that effects of 1 Hz stimulation, although milder, may persist for longer after cessation of treatment than the effects of 10 Hz stimulation. We discuss the longitudinal FC changes observed here in rats in the context of FC changes reported in human rTMS literature (healthy and patient populations). Although to directly extrapolate findings from healthy naïve rats to patients may be tenuous at this early stage of investigation, our findings support the existing use of weekly maintenance treatments in 10 Hz treatment of depression [see review, [52]].

*Functional connectivity changes in 10 Hz group*

In line with previous studies showing increased excitability and induction of LTP following high-frequency rTMS, we found that daily 10 Hz LI-rTMS potentiated FC in the C1, C2 and C3 networks. Our results of increased FC in C1 and C2 are in agreement with findings from previous human studies reporting that rTMS increased FC of the DMN in patients with multiple system atrophy [53] and also of the cortico-striatal-thalamic network in patients with stroke [54] and in maladaptive emotion regulation [55–57]. Additionally, our finding of increased C3 FC to the left hippocampus, somatosensory cortex and striatum is comparable to the increased FC detected between the stimulated parietal cortex and the hippocampus following 20 Hz rTMS in healthy adults [58] and the increased activity of the stimulated somatosensory cortex and the basal ganglia following 5 Hz rTMS in patients with focal dystonia [59], although the increased FC observed here is in the contralateral hemisphere.

Interestingly, within C3 we also found decreased FC in several regions, which is in contrast with previous reports of increased activity in the temporal cortex and hippocampus after two sessions of 20 Hz rTMS in humans [60]. However, the decrease in C3 FC to some brain regions is potentially due to a change in their resting activity making them more synchronous to other components and therefore less synchronous to C3. There is considerable overlap in the brain regions showing a decrease in FC to C3 and an increase in FC to other networks, e.g., increase in C1 FC to the left thalamus ($z = 40–41$), increase in C2 FC to the right thalamus ($z = 33–34$), and increase in C2 FC to the right striatum ($y = 84–86$).

In contrast to the significant changes observed after one day [21] and after 15 days of stimulation, FC within all networks after stimulation cessation was not significantly different from baseline. However, changes in FC compared to SD14 were more widespread and significant on PSD20 than on PSD7, showing that the change in

FC observed on SD14 compared to baseline decreased gradually, back to baseline, after daily stimulation was stopped. Our finding is relevant in refining an optimal strategy for maintenance treatments, particularly for depression in which 10 Hz stimulation is an approved treatment regime (although iTBS has recently also been approved [61,62]). Most studies of rTMS treatment for depression focus on immediate and long-term effects, with only a few behavioural studies investigating the efficacy of "top-up" or maintenance rTMS delivery to prevent relapse [e.g., Refs. [5,63–65]]. However, because these are performed in patients, there is considerable variation within such studies and establishing a reliable protocol is difficult. Our studies suggest that weekly maintenance stimulation delivery may be appropriate, but future studies should investigate if an optimal time can be identified for delivering "top-up" rTMS to maintain FC changes in humans.

*Functional connectivity changes in 1 Hz group*

Overall, 1 Hz stimulation had less widespread effects on FC than 10 Hz stimulation, which was also observed in our previous study looking at the immediate effects of one 10 min LI-rTMS session [21]. Additionally, in the present study, daily 1 Hz stimulation mostly attenuated FC. The differential effect-size and effect-direction between low- and high-frequency rTMS has been observed in previous studies [see review [66]]; induction of rTMS-related changes is known to be less likely with low- than with high-frequency rTMS, and when changes are induced, the effect-direction is negative following low-frequency rTMS and more frequently positive following high-frequency rTMS.

There was a significant decrease in C2 FC to the left and C4 FC to the right somatosensory cortex on SD14. Similarly, a bilateral decrease in activity in somatosensory cortex following 1 Hz rTMS in healthy volunteers was observed by Min et al. [67]. However, in contrast to our findings, they observed more prominent and longer-lasting changes in the contralateral compared to the ipsilateral somatosensory cortex. Nevertheless, this difference might be related to the state dependency of rTMS effects [e.g., reported in Ref. [68]] as Min and colleagues [67] used a finger-tapping task instead of resting-state. Other rTMS studies have reported exclusively ipsilateral effects of 1 Hz stimulation on the somatosensory cortex [69,70], but have used different stimulation and detection methodologies, making comparisons difficult.

Additionally, we observed a significant decrease in FC of the prelimbic cortex (homologous to human medial prefrontal cortex) to the basal ganglial network (C3), consistent with human literature showing attenuated FC between these two regions in healthy individuals following 1 Hz stimulation [71]. Similarly, in patients with depression, 1 Hz rTMS has been shown to decrease activity in the prefrontal cortex and the basal ganglia while 10 Hz rTMS, most often used to alter aberrant connectivity in depression [see review [72]], increased activity within these regions [73,74]. Surprisingly, both frequencies are reported to have similarly beneficial outcomes in patients, with about half of the participants in each group responding to treatment [75]. One study even reported that patients who benefit from one frequency might worsen from the other [73]. This might be explained by the observation that patients can exhibit either hypoconnectivity between the ventromedial prefrontal cortex and ventral striatum, or hyperconnectivity between the dorsal prefrontal cortex and dorsal caudate [76]. Therefore, pre-treatment FC within this network might play an important role in patients' antidepressant response to 1 Hz and 10 Hz rTMS [77].

Upon cessation of stimulation, C2 FC increased back to baseline, whereas the decrease in FC was sustained on PSD7 within C3 and even on PSD14 within C4. The continuous decrease in C4 FC following stimulation cessation is surprisingly strong and may be related to either a decrease in excitatory circuits or an increase in inhibitory mechanisms. Previous animal studies have consistently shown increases in inhibitory circuit function a few hours after 1 Hz stimulation, but there are few studies that measure changes occurring days or weeks after stimulation. One study in rats demonstrated increasing expression of cortical markers of inhibitory neurotransmission over seven days after a single 70 min 1 Hz stimulation [78]. Taken together, our findings of decreased C2, C3 and C4 FC suggest that 1 Hz stimulation may have a sustained effect on the RSN by increasing inhibitory neurotransmission. LI-rTMS at 1 Hz may therefore be a useful tool for inducing long-term changes in brain function and warrants further investigation.

*Changes in neurometabolite concentrations induced by 10 Hz and 1 Hz stimulation*

Similar to our FC results, 10 Hz stimulation resulted in significant changes in neurometabolite concentrations, whereas 1 Hz effects were subtler but more sustained. Previous rodent studies also suggested that repeated 1 Hz stimulation has limited effects on cortical markers of neuroplasticity [79] and induces only non-significant decreases in gene expression in the cortex [80]. We are cognisant that the difference in effect size and persistence of 10 Hz and 1 Hz LI-rTMS might be related to differences in the total number of pulses applied (6000 vs 600 pulses respectively) [81]. For example, a systematic study found that 600 pulses of 10 Hz stimulation had an excitatory effect but 1200 pulses had no effect [81].

To gain insight into the effect of LI-rTMS on excitatory and inhibitory networks within the sensorimotor cortex, we measured Gln and Glu, which have been positively correlated with various rTMS-measures of cortical excitability [9,14,15]. We also measured Glx (representing the sum of Gln + Glu) and Glu/Gln ratio to support the Gln and Glu data, as well as GABA, the main inhibitory neurotransmitter in the brain. *Post hoc* analyses revealed a significant but transient increase in Gln and non-significant increase in Glu, Glx and GABA following seven days (SD7) of 10 Hz stimulation. However, after an additional seven days of stimulation (SD14), levels of all four compounds returned to baseline. The noteworthy drop in metabolite levels observed on SD14 appears contradictory to the maintained increase in FC shown by our rs-fMRI data at this time point. A possible explanation is that the increased excitability induced by 10 Hz LI-rTMS triggers homeostatic and/or metaplastic mechanisms to maintain a balance of excitation/inhibition in the brain [82,83] (previously shown in neuronal cultures [84] and in rats [78,85]), but without disrupting the increases in FC. Also, the MRS data reflect changes in the sensorimotor cortex only while FC changes were found in other brain regions as well. Nonetheless, the engagement of homeostatic control by 10 Hz LI-rTMS is consistent with the transient increase in Glu/Gln ratio on PSD7 and short duration of FC changes, which were no longer detected a week after stimulation cessation. The implication is that 10 Hz may initially induce stronger effects in neural circuits than 1 Hz, but as a result, recruits compensatory mechanisms which limit the duration of LI-rTMS effects. By contrast, 1 Hz, which has more subtle effects on FC and neurometabolites, elicits more persistent effects with a sustained decrease in cortical Gln, Glu and Glx seven days after cessation of stimulation (PSD7) and changes in FC compared to baseline in the C4 network persisting up to the last timepoint studied (PSD14).

Interestingly, the persistent decrease in GABA levels after cessation of 10 Hz stimulation, despite a lack of change in FC, is also consistent with a long-term facilitatory effect of 10 Hz stimulation acting via depression of GABAergic neurotransmission [see review 86]. Given that inhibitory interneurons control the activity and

excitability of cortical principal neurons [87], previous studies have suggested that depression of inhibitory neurotransmission, as seen here, could facilitate associative plasticity (e.g., for improving learning and memory) in cortical networks [86]. This makes rTMS an attractive therapeutic intervention for several neuropsychiatric conditions associated with changes in inhibitory synaptic plasticity and excitation/inhibition-balance leading to behavioural and cognitive dysfunction [88], for example in schizophrenia [89,90] and autism [91].

The results of previous human MRS/rTMS studies are generally consistent with our findings, although use of healthy animals, the different number of stimulation sessions, as well as differences in intensity and frequency, preclude a direct comparison. Long-term (days to weeks) high-frequency rTMS to the prefrontal cortex increased cortical Gln and Glu levels in healthy volunteers [92], in patients with depression [93,94] and in patients with schizophrenia [95]. Interestingly, experiments in humans focussing on immediate effects of rTMS detect a reduction in inhibitory synaptic interactions following high-frequency rTMS using motor evoked potentials (MEP) and paired-pulse protocols [reviewed in Ref. [96]]. More recently, combined TMS paired-pulse and MRS studies show that the amount of intracortical inhibition does not correlate with the global levels of GABA in the primary motor cortex but may instead be linked to cortical glutamate levels [97]. Taken together, studies in humans and in rodents concur that rTMS induces complex changes in inhibitory and excitatory circuits that evolve over time and may involve frequency-specific effects on different cell types [reviewed in Ref. [98]]. Our findings suggest that LI-rTMS in rodents induces changes that clearly reflect those occurring in humans following rTMS, and therefore provide a unique opportunity to combine non-invasive and invasive methods in the investigation of rTMS effects on neural circuitry.

Nevertheless, this is the first exploratory study of longitudinal effects of repeated LI-rTMS on rodent neurometabolites, and researchers interested in, for example, more subtle effects should consider dedicating more time to collect spectra with higher SNR. This may reduce the observed variability of some low concentration metabolites such as GABA and Gln. Additionally, researchers interested in the effects of rTMS on GABA could consider using MEGA-PRESS [99] or PRESS with TE (echo time) optimized for that metabolite [100].

## Conclusion

Information about the duration of the after-effects of rTMS therapy is vital for the development and improvement of rTMS use as a treatment in a clinical setting. Here, we present the first longitudinal rs-fMRI/MRS investigation of the duration of FC and neurometabolic changes induced by repeated LI-rTMS delivery. Our work confirms the frequency-specific effects of LI-rTMS and further suggests that effects of 1 Hz stimulation, although milder, may persist longer after cessation of treatment compared to those of 10 Hz stimulation. This study provides a framework to use non-invasive brain imaging to explore the duration of rTMS effects on resting brain activity in animal models of neurological and neuropsychiatric disorders such as depression for development and translation of optimised protocols to human patients. Further studies in animals and humans are warranted in effort to investigate potential prolongation of FC effects through maintenance or “top-up” rTMS sessions weeks or months after the first set of treatment.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.brs.2019.06.028.

## Author contributions

BJS and JR contributed to the experimental background and design. BJS conducted the experiments, analysed the results and wrote the first version of the manuscript. KWF established the MRI acquisition protocol and provided troubleshooting and methodological advice on acquiring and analysing the imaging data. All authors revised and proofed the manuscript.

## Conflicts of interest

The author(s) declare no financial and/or non-financial conflicts of interests associated with this publication.

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