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Accelerated low-intensity rTMS does not rescue anxiety behaviour or abnormal connectivity in young adult rats following chronic restraint stress

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Abstract

Currently approved repetitive transcranial magnetic stimulation (rTMS) protocols for the treatment of major depressive disorder (MDD) involve once-daily (weekday) stimulation sessions, with 10 Hz or intermittent theta burst stimulation (iTBS) frequencies, over 4–6 weeks. Recently, accelerated treatment protocols (multiple daily stimulation sessions for 1–2 weeks) have been

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Appendix A. Supplementary data

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increasingly studied to optimize rTMS treatments. Accelerated protocols might confer unique advantages for adolescents and young adults but there are many knowledge gaps related to dosing in this age group. Off-label, clinical practice frequently outpaces solid evidence as rigorous clinical trials require substantial time and resources. Murine models present an opportunity for high throughput dose finding studies to focus subsequent clinical trials in humans. This project investigated the brain and behavioural effects of an accelerated low-intensity rTMS (LI-rTMS) protocol in a young adult rodent model of chronic restraint stress (CRS). Depression and anxiety-related behaviours were induced in young adult male Sprague Dawley rats using the CRS model, followed by the 3-times-daily delivery of 10 Hz LI-rTMS, for two weeks. Behaviour was assessed using the Elevated Plus Maze and Forced Swim Test, and functional, chemical, and structural brain changes measured using magnetic resonance imaging techniques. CRS induced an agitated depression-like phenotype but therapeutic effects from the accelerated protocol were not detected. Our findings suggest that the age of rodents may impact response to CRS and LI-rTMS. Future studies should also examine higher intensities of rTMS and accelerated theta burst protocols.

Keywords

rTMS; Chronic stress; Anxiety; Animal model; MRI; Behaviour

1. Introduction

Repetitive Transcranial Magnetic Stimulation (rTMS) is a non-invasive brain stimulation technique that is FDA approved for treatment-resistant major depressive disorder (MDD), obsessive-compulsive disorder, and migraine (Horvath et al., 2010). While the therapeutic mechanism of action of rTMS remains poorly understood, recent neuroimaging studies in patients with MDD have shown that rTMS treatment is associated with widespread changes in brain connectivity, and that the magnitude of specific connectivity changes is correlated with symptom improvement (Philip et al., 2018). Currently approved rTMS protocols for the treatment of MDD involve once-daily (weekday) stimulation sessions, with 10 Hz or intermittent theta burst stimulation (iTBS) frequencies, over 4–6 weeks. More recently, accelerated treatment protocols (multiple daily stimulation sessions for 1–2 weeks) have been studied in the belief that increasing the dose will improve outcomes (Baeken et al., 2021; Baeken et al., 2020; Cole et al., 2020; Williams et al., 2018). However, there is a lack of clear evidence for a biological benefit of accelerated over standard protocols (Fitzgerald et al., 2018; Modirrousta et al., 2018). A better understanding of the behavioural and brain outcomes associated with rTMS will support optimisation of dosing and treatment parameters required to improve patient outcomes.

Off-label, clinical practice frequently outpaces evidence as rigorous clinical trials require substantial time and resources. Murine models offer a cost-effective opportunity for high throughput dose finding studies to focus subsequent clinical trials in humans. MRI-based experiments have shown that changes in brain connectivity in rodents following low-intensity magnetic fields (LI-rTMS; to maximise focality (Rodger et al., 2012)) reflect those observed in healthy humans after conventional TMS (Seewoo et al. 2018, 2019b). LI-rTMS has also been applied in rodent models of depression with promising behavioural results:

LI-rTMS successfully rescued behavioural and neurological symptoms in a mouse model of depression (Heath et al., 2018), and a study in adolescent rats suggested a benefit of low-intensity 10 Hz accelerated compared to standard rTMS protocol on depression-like behaviours (Seewoo et al., 2021b). However, to date there have been no studies correlating behavioural and brain-based outcomes of rTMS in an animal model of depression.

For the first time, here we report repeated measures of behaviour and brain connectivity in young adult rats subjected to chronic restraint stress (CRS), followed by treatment with an accelerated 10 Hz low-intensity rTMS protocol. CRS is a well-established model of anxiety and depression in rodents and is accompanied by changes in brain connectivity that reflect those reported in human subjects with depression. Furthermore, changes in behaviour post CRS in rodents are associated with changes in connectivity (Seewoo et al., 2020). We chose to study accelerated rTMS because recent clinical studies suggest that an accelerated delivery that provides a higher dose of stimulation may be as effective as standard delivery and may have advantages in some populations (Baeken et al., 2021; Baeken et al., 2020; Cole et al., 2020; Seewoo et al., 2021b; Williams et al., 2018). Our results show that although CRS successfully induced an agitated depression-like phenotype that was detected in behavioural and brain imaging measures, two weeks of accelerated 10 Hz LI-rTMS did not influence behaviour, brain connectivity, hippocampal volume or neurotransmitter levels. Our results are discussed in the context of possible confounders of age, phenotype as well as rTMS intensity and frequency.

2. Materials and methods

2.1. Animals

Fifty-six male Sprague Dawley rats (7–8 weeks old; 290±47 g) were sourced from the Animal Resources Centre, Western Australia. Rats were habituated to the animal care facility for one week prior to experiments. All animals were group-housed (two rats/cage) in a temperature-controlled, standard 12 h light/dark cycle environment with food and water provided *ad libitum* except where specified in the methods.

2.2. Study overview

The experimental protocol was approved by the University of Western Australia Animal Ethics Committee (RA/3/100/1640) and was in accordance with the National Health and Medical Research Council's *Australian Code for the Care and Use of Animals for Scientific Purposes (8th* Edition, 2013).

Animals were randomly assigned to one of three groups: accelerated active treatment (n = 20 per group), accelerated sham treatment (n = 20 per group), or no treatment (depression-induced control group; n = 16 per group). As batches of animals completed the experiment step-wise, each condition was equally allocated within batches (i.e. random assignment of four animals to each condition in batches of 12 animals). All animals underwent chronic restraint stress for 2.5 h per day for 13 days. Following CRS, animals in the treatment groups received 3 sessions of active or sham 10 Hz stimulation per day (spaced 1 h apart), for 5 consecutive days per week, for 2 weeks. Each stimulation session consisted of 10 min

of 10 Hz low-intensity stimulation, in line with previous animal studies (Makowiecki et al., 2018; Moretti et al., 2020; Poh et al., 2019; Seewoo et al. 2018, 2019b, 2021a, 2021b). For all animals, behavioural tests and magnetic resonance imaging procedures were conducted at baseline, after the chronic restraint stress period, midway through treatment, post-treatment, and at one-week and two-week follow-up time points (Fig. 1). Behavioural testing and imaging were conducted over a four-day period. This testing period was conducted before moving onto the next stages of the experiment, with the exception of the mid-treatment timepoint (comprised of two days), where the testing period overlapped with the fifth and sixth days of rTMS delivery.

2.3. Chronic restraint stress

Depression-like behaviours were induced using a Chronic Restraint Stress (CRS) model (see Ulloa et al. (2010)), as validated in Seewoo et al. (2020). Rats were placed in individual transparent acrylic tubes for 2.5 h each day, for 13 consecutive days.

2.4. Repetitive transcranial magnetic stimulation

Stimulation was delivered at 10 Hz using a custom-built round coil (8 mm inner diameter x 16.2 mm outer diameter x 10 mm height; 460 turns of 0.25 mm diameter copper wire; 6.1 Ω resistance). The coil was connected to a pulse generator and produced a magnetic field intensity of 24 mT at the coil surface and 13 mT at the surface of the cortex (Grehl et al., 2015; Seewoo et al. 2018, 2019b). Sham stimulation was delivered with the pulse generator switched off, acting as a handling control. Our goal was to position the coil with the maximum induced electric field over the left prefrontal cortex: Animals were placed on the investigator's lap, and the coil was placed flat against the dorsal-lateral (left) side of the animal's head, with the rostral-most edge of the coil positioned behind the left eye. In this way, the area immediately underneath the coil winding (with the maximum induced electric field) targeted the left prefrontal cortex. Because of the circular shape of the coil, other more caudally located brain regions also received stimulation although at lower intensity (modelled in (Seewoo et al., 2018). Coils were manually held in the appropriate position relative to the animal's head throughout the stimulation period because the animals were awake and lightly restrained and made occasional head movements. Each treatment session was conducted during the afternoon, commencing between 12:00–13:30.

2.5. Behavioural testing

2.5.1. Elevated Plus Maze—Animals first underwent the Elevated Plus Maze (EPM) test (see Walf and Frye (2007) for full protocol) to assess the presence of anxiety-related behaviours. Animals were placed in the centre of a plus-shaped maze, facing an open arm, and allowed to explore the maze for 5 min.

2.5.2. Sucrose preference test—The Sucrose Preference test was conducted following the EPM, but yielded unreliable results as previously reported (Seewoo et al., 2020). Protocol is described in supplementary information.

2.5.3. Forced Swim Test—The Forced Swim Test (FST) was conducted last in the sequence of behavioural tests to evaluate learned-helplessness (Slattery and Cryan, 2012).

Animals were individually placed for 6 min in white, opaque 20 L buckets (height = 41 cm, diameter = 28 cm) filled with water (23–25 °C) to a depth of 30 cm. Animals were exposed to the test conditions in a pre-test (see Slattery and Cryan (2012)).

2.5.4. Analysis of behavioural videos—For the EPM and FST, behaviour was recorded using a GoPro Hero 7 (GoPro, Inc.) camera and the footage analysed offline (full 5 min for the EPM, the first 5 min for the FST) by a trained experimenter blind to condition and timepoint. For the EPM, exploration was determined through the number of exits and time spent in the open and closed arms. Rearing and grooming behaviours were also measured to quantify stress responses. For the FST, the video was split into 5 s segments. Each segment was analysed to determine the predominant behaviour. Behaviours were classified as either climbing, swimming, or immobility. Latency to the first segment with predominant immobility was also determined.

2.6. Magnetic resonance imaging

2.6.1. MRI procedure—Imaging sessions were conducted in the two days following the Forced Swim Test. Animals were placed in an induction box containing 4% Isoflurane in medical air, then transferred to the MRI machine as described previously (Seewoo et al., 2020). Isoflurane was then slowly reduced to 2%, at which point a 0.05 mg/kg bolus dose and continuous 0.15 mg/kg/hr infusion of medetomidine were delivered subcutaneously. The concentration of isoflurane was gradually decreased to 0.5–0.75% for resting-state functional MRI (rs-fMRI) data collection. At the conclusion of the imaging session, animals were returned to their home cage and a 0.15 mg/kg subcutaneous injection of atipamezole was administered as a medetomidine reversal agent. Although isoflurane has been shown in some studies to be an antidepressant, the concentrations we used were well beneath those showing evidence of an antidepressant effect (Antila et al., 2017).

2.6.2. MRI parameters—Images were acquired using a 9.4 T (400 MHz; H-1) Bruker Biospec 94/30 US/R pre-clinical MRI machine (Bruker BioSpin GmbH, Germany). A BGA-12SHP imaging gradient system and Avance III console were operated using ParaVision 6.0.1 software. An 86 mm-diameter volume transmit coil, and rat brain surface quadrature receiver head coil were also used. To acquire the scans, we followed a previous imaging protocol, as detailed in Seewoo et al. (2018). First, an anatomical localiser scan was obtained, followed by a multi-slice 2D rapid acquisition with relaxation enhancement (RARE) sequence for three T2-weighted anatomical scans (21 coronal slices, 21 axial slices, 20 sagittal slices, 1 mm thickness; TE = 33 ms, TR = 2500 ms; 280 × 280 matrix; 0.1 × 0.1 mm^2 pixel size). For 1H-magnetic resonance spectroscopy, a point resolved spectroscopy (PRESS) sequence consisting of a 90° pulse followed by two 180° pulses and water suppression was obtained (64 averages; TE = 16 ms, TR = 2500 ms), using a single voxel (3.5 × 2 × 6 mm^3) placed over the left sensorimotor cortex (for consistency with locations chosen in human studies; as discussed in Seewoo et al. (2020)). Rs-fMRI data was acquired using the single-shot gradient echo planar imaging sequence (21 coronal slices, 1 mm thickness; TE = 11 ms, TR = 1500 ms; 94 × 70 matrix; 0.3 × 0.3 mm^2 pixel size; 90° flip angle; 300 vol; first order automatic ghost correction; 300 kHz receiver bandwidth).

2.6.3. Data analysis—All MRI data was processed and analysed as previously described in Seewoo et al. (2020).

All rs-fMRI data was pre-processed and analysed using FSL v5.0.10 (Functional MRI of the Brain (FMRIB) Software Library) (Jenkinson et al., 2012) and following the data analysis pipeline detailed in Seewoo et al. (2021c). Multi-subject temporal concatenation group-ICA and FSL dual regression analysis was conducted (see supplementary information), followed by seed-based analysis using the atlas mask for the cingulate cortex. Higher-level analysis was carried out using OLS (ordinary least squares) simple mixed-effects (Beckmann et al., 2003; Woolrich, 2008; Woolrich et al., 2004) in atlas space. Z (Gaussianised T/F) statistic images were thresholded non-parametrically using clusters determined by $Z > 2$ and a (FWE-corrected) cluster significance threshold of $p = .05$ (Worsley, 2001).

To assess hippocampal volume, the three T2-weighted anatomical (coronal, sagittal and axial) data were pre-processed as described in Seewoo et al. (2020) and then registered to the high-resolution Sprague Dawley brain atlas (no downsampling). Atlas masks for bilateral hippocampus (including cornu ammonis and dentate gyrus) and whole-brain (grey matter, white matter and cerebrospinal fluid) were transformed to each individual animal's anatomical space. The 'fslstats' command was used to extract bilateral hippocampal volumes and whole-brain volumes from each of the three planes. Hippocampal and whole-brain volumes from the three planes were averaged for each animal scan session. Hippocampal volume was normalised to whole brain volume to adjust for differences in head size, as previously described by Welniak–Kaminska et al. (2019). All hippocampal volume results presented here are expressed as a percentage of whole-brain volume.

1H-MRS data were analysed in LCModel ("Linear Combination of Model spectra" version 6.3–1 L) (Provencher, 2001) 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. As a measure of reliability of metabolite concentrations, Cramér-Rao lower bound (CRLB) values were calculated by LCModel and reported as percent standard deviation of each metabolite. The metabolites of interest were γ-aminobutyric acid (GABA), glutamate (Glu), glutamine (Gln), and combined glutamate-glutamine (Glx). All 1H-MRS results are expressed as a ratio to tCr (total creatine = Cr + PCr) spectral intensity, the simultaneously acquired internal reference peak (Block et al., 2009; Walter et al., 2009; Xu et al., 2013).

2.7. Statistical analysis

Statistical analysis was conducted using SPSS (v. 21, IBM Corporation, Armonk, New York, USA), RStudio (v. 4.0.2, RStudio Team, Boston, Massachusetts, USA), and DABEST (via https://estimationstats.com), with figures created and directly downloaded from https://estimationstats.com.

Paired student's t-tests and Wilcoxon signed ranks tests (for non-parametric data) were used to assess the effect of the chronic restraint stress model on all behavioural measures and MRI outcomes compared to baseline. Mixed model analysis of variance (ANOVA) was used to test the effect of treatment group and timepoint for both behavioural tests and change in

functional connectivity, hippocampal volume, and metabolite levels. Visual inspection of QQ plots was used to assess normality. All variables met the assumption of normal distribution except the following behavioural measures: open arm exit count, time spent in open arms, time spent grooming, and climbing count. These variables were then log transformed to meet normality. Follow-up Tukey's HSD and Games-Howell *post hoc* tests were used to account for multiple comparisons.

Estimation statistics based on confidence intervals (CI) and 5000 bootstrap samples were conducted to further assess the data over time within each treatment group. *P* values represent the likelihood of observing the effect sizes, with Cohen's d used to measure effect size (with the exception of the behavioural test multi-paired plots, which instead used the paired median difference due to a large number of animals recording zero counts and thus a non-normal distribution).

We note that some data points were excluded from the analyses due to technical failures, including failure of the MRI machine and associated equipment (heating blankets, monitoring equipment), camera recording failure, anaesthetic failure, and the limitations of statistical packages in dealing with null values. A full outline of these exclusions is provided in the supplementary material. All measurements are presented as mean±SEM unless otherwise specified, with the level for statistical significance set at $p < .05$.

3. Results

3.1. Baseline vs Post-CRS

We first compared behaviour and brain changes in rats at baseline and immediately following CRS to characterise the impact of the CRS intervention.

In the Elevated Plus Maze, there was a significant increase in the number of exits out of both the closed arms (Baseline $M = 6.88$, $SE = 0.59$; Post-CRS $M = 9.33$, $SE = 0.48$; $n = 52$; $Z = -3.375$, $p = .001$) and open arms (Baseline $M = 0.52$, $SE = 0.15$; Post-CRS $M = 1.02$, $SE = 0.19$; $n = 50$; $Z = 2.099$, $p = .036$) following CRS. There was also a significant decrease in the total amount of time spent in the closed arms (Baseline $M = 220.37$, $SE = 4.88$; Post-CRS $M = 193.21$, $SE = 6.96$; $n = 52$; $t(51) = 3.771$, $p < .001$), and a significant increase in total time spent in the open arms (Baseline $M = 3.72$, $SE = 1.05$; Post-CRS $M = 9.64$, $SE = 2.18$; $n = 50$; $Z = -2.562$, $p = .010$). Additionally, there was an increase in number of times the animals exhibited grooming behaviours (Baseline $M = 2.56$, $SE = 0.30$; Post-CRS $M = 3.44$, $SE = 0.30$; $n = 50$; $Z = -2.299$, $p = .022$). However, there were no significant changes in total time spent grooming, number of times demonstrating rearing behaviour, or total time spent rearing. In the Forced Swim Test, there was a significant decrease in swimming compared to baseline levels (Baseline $M = 32.23$, $SE = 2.14$; Post-CRS $M = 25.43$, $SE = 2.09$; $n = 35$; $t(34) = 3.039$, $p = .005$; Fig. 2). There was also an increase in immobility and a reduced latency to the first immobility score, although these differences were not significant. There were no significant differences found for climbing behaviour.

Assessment of resting-state fMRI data using seed-based analysis identified a reduction in functional connectivity between the cingulate cortex and a range of regions following CRS (t (47) = 8.112, p < .001; Fig. 3). Furthermore, the percentage of total hippocampal volume did not change immediately following CRS compared to baseline levels (Baseline M = 5.87%, SE = 0.005; Post-CRS M = 5.87%, SE = 0.008; p = .356). Individual left or right hippocampal volumes also did not change for post-CRS timepoints (Left M = 2.90%, SE = 0.004, p = .247; Right M = 2.97%, SE = 0.004; n = 49; p = .630). There were also no significant changes in any measured neurometabolite levels following CRS (p >.05) (not shown; see supplementary material information).

3.2. rTMS treatment and long-term outcomes

Having confirmed that CRS had induced brain and behavioural changes, animals were randomly allocated to accelerated rTMS treatment, accelerated sham treatment, or no intervention for 2 weeks. Behavioural and MRI outcome measures were monitored weekly during, and for 2 weeks following treatment.

3.2.1. Behaviour—There was no clear effect of LI-rTMS on behaviour following CRS (Fig. 4). In the Elevated Plus Maze, the mixed model ANOVA found a significant interaction between treatment group and timepoint for total time spent grooming (F (10,115) = 1.99, p = .041), with significant simple main effects found in the sham group between mid-treatment and post-treatment timepoints (p = .007) and between the sham and control groups at the mid-treatment timepoint (p = .049). No other simple main effect comparisons were significant (Fig. 4). There were no interactions found for the other variables, however a main effect of timepoint was found for total time spent in closed and open arms, total exits from closed and open arms, and total count of grooming behaviours. No variables showed an effect of treatment on behaviour in the EPM.

For the Forced Swim Test, there was a significant interaction between treatment group and timepoint for swimming (F (7.20,112.94) = 2.18, p = .039), with significant simple main effects found in the active group between baseline and mid-treatment timepoints (p = .001), and in the sham group between the baseline and week 2 follow-up (p = .026), post-treatment and week 2 follow-up (p = .006), and week 1 and week 2 follow up timepoints (p = .048) (Fig. 4). However, there were no significant interactions or main effects found for the other variables.

3.2.2. Resting-state fMRI—Using seed-based analysis of fMRI data, the mixed model ANOVA found a significant main effect of timepoint on functional connectivity to the cingulate cortex (F (5254.77) = 13.03, p = < .001), but no main effect of treatment or interaction between the two variables suggesting that brain connectivity changed over time in all rats. Accelerated LI-rTMS did not have an impact on brain connectivity over time (Fig. 5).

3.2.3. Hippocampal volume—The mixed model ANOVA found a significant main effect of timepoint on total hippocampal volume (F (5260.52) = 3.51, p = .004), but no main effect of group or interaction between the two variables. Similarly, when left and

right hippocampi were analysed separately, the mixed model ANOVA found a significant main effect of timepoint for both left (F (5260.97) = 2.47, $p = .033$) and right hippocampal volumes (F (5260.51) = 3.41, $p = .005$), but no main effect of group or interaction between the two variables. This data suggests that hippocampal volume slightly increased over time following CRS and was not affected by accelerated LI-rTMS (Fig. 6).

3.2.4. ^{1}H magnetic resonance spectroscopy—The levels of γ-aminobutyric acid (GABA), glutamate (Glu), glutamine (Gln) and combined glutamate-glutamine (Glx) were measured in the sensorimotor cortex across all timepoints and determined as concentrations relative to tCr. A mixed model ANOVA was used to compare the levels across all conditions and timepoints. There was a significant main effect of timepoint on the levels of Glu (F (5264.98) = 3.22, $p = .008$), but not for GABA, Gln, or Glx ($p > .05$). There was no main effect of group or significant interaction found for any of these recorded metabolites. The data suggest that there were small fluctuations in Glu over time that were not related to treatment with accelerated LI-rTMS (Fig. 7).

4. Discussion

rTMS is a promising tool for treating patients with neuropsychiatric conditions, however there is a need to better understand the dosing parameters in order to develop optimal treatment protocols. Our aim in the present study was to evaluate the long-term effects of an accelerated LI-rTMS protocol on brain and behavioural outcomes in young adult rats subjected to chronic restraint stress (CRS). The rats unexpectedly displayed hyperactivity and an agitated depression-like behavioural phenotype in response to CRS, which was associated with a decrease in cingulate cortex connectivity. However, treatment with accelerated 10 Hz LI-rTMS did not alter behaviour or brain-based measures. Our findings contrast with the positive outcomes previously reported after accelerated LI-rTMS in younger adolescent rats (6–7 weeks, at the start of the experiment (Seewoo et al., 2021b)). One possible interpretation is that the response to CRS and potentially LI-rTMS depends on the age and/or weight and size of the animals, and these factors are discussed in detail at the end of the discussion. Another possibility is that the variability across our experiments matches the variability in symptoms and rTMS treatment outcomes reported in human patients with depression, suggesting that our database of preclinical experiments may provide a valuable resource for investigating predictive biomarkers of rTMS efficacy.

4.1. Decreased functional connectivity and behavioural agitation following chronic restraint stress

An unexpected finding in our experiments was that the changes induced by CRS in young adult rats, both in terms of brain connectivity and behaviour do not conform to standard presentations of anxiety and depression-like behaviours. Abnormal functional connectivity is a common characteristic of depression and anxiety in human patients (Mulders et al., 2015; Kaiser et al., 2015; Kim et al., 2011; Hilbert et al., 2014), with the cingulate cortex generally showing increased functional connectivity with regions in the limbic system, resulting in deficits in moderating and regulating the processing of emotion in mood disorders (Greicius et al., 2007; Davey et al., 2012; Rolls et al., 2019; Mulders et al.,

2015; Connolly et al., 2013). Similarly, following chronic restraint stress in rodents, we and others have previously demonstrated decreased functional connectivity within the salience and interoceptive networks, and hyperconnectivity of multiple regions to the cingulate cortex (Seewoo et al., 2020). However, in the current study, we instead identified a decrease in connectivity between the cingulate cortex and several brain regions, similar to findings in human patients with trauma and/or anxiety (Kennis et al., 2015; Chen et al., 2019; Chang and Yu, 2018; Hahn et al., 2011).

Changes in behaviour also support a non-standard response to CRS. In the EPM, rats spent more time in the open arms, suggesting a reduction in anxiety, however this was combined with increased movement across all arms, suggesting hyperactivity and agitation. Similarly in the FST, animals showed both increased immobility suggesting learned helplessness, but they also had a significant increase in climbing behaviour, suggesting agitation. Taken together, these behaviours may reflect aspects of a clinical presentation of depression and anxiety with agitation.

Hyperactivity has been reported in other animal models of depression, such as olfactory bulbectomy in mice (Heath et al., 2018; Masini et al., 2004; Wang et al., 2007), and is a common clinical symptom of depression in some human patients (Serra et al., 2019; Iwanami et al., 2015; Angst et al., 2009). Moreover, psychomotor agitation is a common symptom of anxiety disorders, particularly when comorbidities of anxiety and mood disorders are present (Kaiser et al., 2021; Zbozinek et al., 2012). Taken together, the MRI and behavioural outcomes suggest that CRS induced an agitated phenotype in our young adult rats, contrasting with previous studies in adolescent and older adult rats showing behaviours predominantly associated with depression (learned helplessness, anhedonia) (Seewoo et al., 2020; Ulloa et al., 2010; Ménard et al., 2016; Chiba et al., 2012; Liu et al., 2016) (see also supplementary info).

4.2. Accelerated LI-rTMS did not affect connectivity, hippocampal volume, metabolites or behaviour

We did not observe any changes in brain or behavioural measures following ten sessions of accelerated LI-rTMS over two weeks. This was surprising given the results from our pilot study suggesting that just one week of accelerated LI-rTMS rescued the effects of CRS (Seewoo et al., 2021b). We cannot rule out that the pilot study produced a false positive result due to a small sample size (n = 5 per group), or that the reduced statistical power due to multiple timepoints in the current study resulted in a false negative, despite the larger group size of n = 12. However, most of the behavioural outcomes had medium to large effect sizes in both the pilot (Seewoo et al., 2021b) and current study (see supplementary material). In contrast, the effect sizes for MRI outcomes were small in the present study, suggesting high variability. An alternative explanation is that the animals used in the present study did not respond to LI-rTMS because of their slightly older age compared to the pilot study cohort (6–7 vs 7–8 weeks upon experiment onset; see below), and/or because accelerated LI-rTMS may not be an effective treatment for the hyperactivity and agitiation that was induced following CRS, even though rTMS has shown benefits in treating anxiety in human patients (Balderston et al., 2020). A further complication in our study is that

significant brain growth in rats continues to occur until approximately 9 weeks of age (Bandeira et al., 2009), with certain regions still developing up to 11 weeks of age (Fu et al., 2013). The continuing maturation of the brain likely underpins the significant effect of timepoint observed across most of the measures in our study and raises the possibility that the combined impacts of CRS, rTMS and concurrent age-related brain maturation might have masked treatment-related changes.

Another consideration is that the experimental design involves repeated behavioural testing which has been reported affect the outcome of EPM and FST (e.g. (Carobrez and Bertoglio, 2005; Schrader et al., 2018)). As animals undergo the test multiple times over several weeks, there is evidence that they learn to adopt specific strategies (e.g. learned helplessness) and become familiar with the conditions of the test (reduced anxiety in the EPM). However there is also evidence that differences in these tests remain detectable compared to control groups, and we previously demonstrated that we could obtain robust responses over time following CRS in both of these tests (Seewoo et al., 2020). Therefore while we acknowledge the limitations and potential confounds of repeated behavioural testing, we suggest that the negative results obtained here are likely due to other factors.

Finally, it is also possible that LI-rTMS was applied at an intensity that was too low to have an effect. In a previous study directly comparing stimulation intensities in mice, only the medium intensity (50 mT – roughly twice as strong as the current LI-rTMS) and high intensity (1 T) protocols caused behavioural changes, while LI-rTMS at 10 mT had no effect (Heath et al., 2018). Nonetheless, it is well established that intensities lower than 24 mT alter brain structure and function in healthy rats, in non-psychiatric mouse models of disease and in drosophila (Dufor et al., 2019; Makowiecki et al., 2014; Poh et al., 2018; Rodger et al., 2012; Sherrard et al., 2018). However, those experiments mostly targeted superficial brain regions such as the cortex and cerebellum (Dufor et al., 2019; Makowiecki et al., 2014; Poh et al., 2018) and higher intensities may be required to stimulate deeper cortical regions and interconnected brain networks at a level sufficient to affect changes in mood (Philip et al., 2018). rTMS in clinical studies involving psychomotor deficits have all used high intensity (>1 T (Baeken et al., 2010; Hoeppner et al., 2010);) and a study that showed increased locomotion following accelerated rTMS in healthy rats used a similar high intensity (El Arfani et al., 2017). Because low and high intensity rTMS call different mechanisms into play, determining the intensity required to induce changes in the brain that are relevant for the treatment of depression may provide insight into the brain mechanisms underpinning neuropsychiatric disorders.

4.3. Influence of age and future directions

We acknowledge that our results do not fully replicate the findings of previously published studies from our own group, nor from other laboratories (Seewoo et al. 2020, 2021b; Ulloa et al., 2010; Ménard et al., 2016; Chiba et al., 2012; Liu et al., 2016). We have discussed the statistical limitations above, and here consider in more depth the possibility that these differences may be associated with age and maturational state of the brain.

We identified un-anticipated differences in the age of the rats used in Seewoo et al. (2020), Seewoo et al. (2021b), and the present study: due to logistical constraints, the animals in

the current study begun procedures at approximately 7–8 weeks of age, while those in Seewoo et al. (2020, 2021b) were aged 6–7 weeks. The difference of 1–2 weeks was also associated with a significant difference in weight (~300 g in current study vs 150–200 g in the former) indicating that the maturity of the two cohorts was very different and may have been associated with sexual maturity differences. Developmental literature suggests that rodents are considered juvenile up to approximately 6 weeks, and reach sexual maturity around postnatal day (PND) 48–60 (i.e. approx. 7–9 weeks) (Brust et al., 2015), however this can vary between individuals, occurring anywhere between PND 40 (5.5 weeks) and 76 (11 weeks) in male Sprague Dawley rats (Lewis et al., 2002). Additionally, significant brain growth continues to occur until approximately 9 weeks of age (Bandeira et al., 2009), with certain regions still developing up to 11 weeks of age (Fu et al., 2013). Our studies may therefore be differentiated based on whether the rats were just before (adolescent; Seewoo et al., 2020, 2021b) or just after (young adult; current study) sexual maturity. The implication is that CRS would have acted on brains in very different maturational states, potentially contributing to the differences in brain and behavioural outcomes. For example, behavioural responses to stress are known to vary non-linearly across developmental periods in rodents: although novelty exploration in the EPM generally decreases with age, adolescent animals are less anxious and more explorative than both juvenile and young-adult animals (Albani et al., 2015; Laviola et al., 2003; Macri et al., 2002). Furthermore, the impact of a stressor on EPM behaviours is stronger in adolescent mice compared to young adult mice (Stone and Quartermain, 1997), which may have contributed to the more robust results we found previously (Seewoo et al., 2021b). Importantly, sexual maturity commonly precedes behavioural maturity (Brust et al., 2015), suggesting that full adulthood may not occur until sometime later. This is particularly important to note, as behavioural responses are more stable in fully mature adult compared to developing animals (Brust et al., 2015). In addition, because the intensity of the magnetic field is reduced with distance, a larger brain and head size would alter the intensity and distribution of stimulation, potentially contributing to the different outcomes.

In summary, there is evidence in the literature that rodents show significant variability in response to stress during the developmental period, potentially explaining why we obtained different outcomes in our experiments. In spite of the current challenges in interpreting these conflicting results, collectively, our past and ongoing studies provide a rich dataset from a significant number of animals of different ages that underwent the same procedures in the same laboratory. We therefore have a unique opportunity to explore the age-related factors that contribute to individual brain and behavioural changes in response to stress and LI-rTMS treatment.

5. Conclusion

Overall, our study suggests that 10 sessions over two weeks of accelerated 10 Hz low-intensity rTMS does not rescue an agitated depression-like phenotype induced in young adult rats by chronic restraint. Our findings highlight that age- and symptom-specific protocols may be required to provide patients with optimal treatment. Further investigation is required to understand the brain-based mechanisms of rTMS to both harness and optimize its therapeutic effects in MDD, particularly in the immature brain.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Declaration of competing interest

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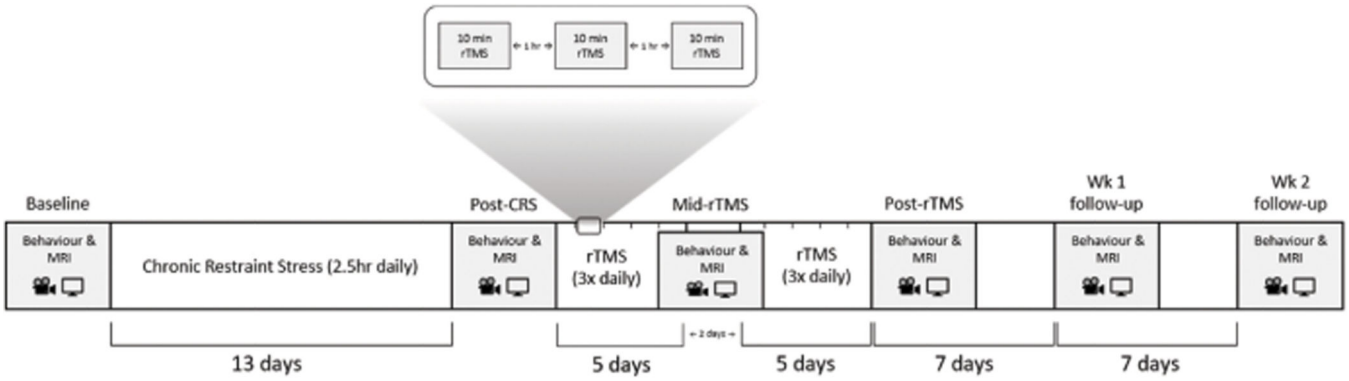

Fig. 1.

Experimental Timeline & Study Design. Behavioural tests and MRI were conducted over a consecutive 4-day period at each timepoint.

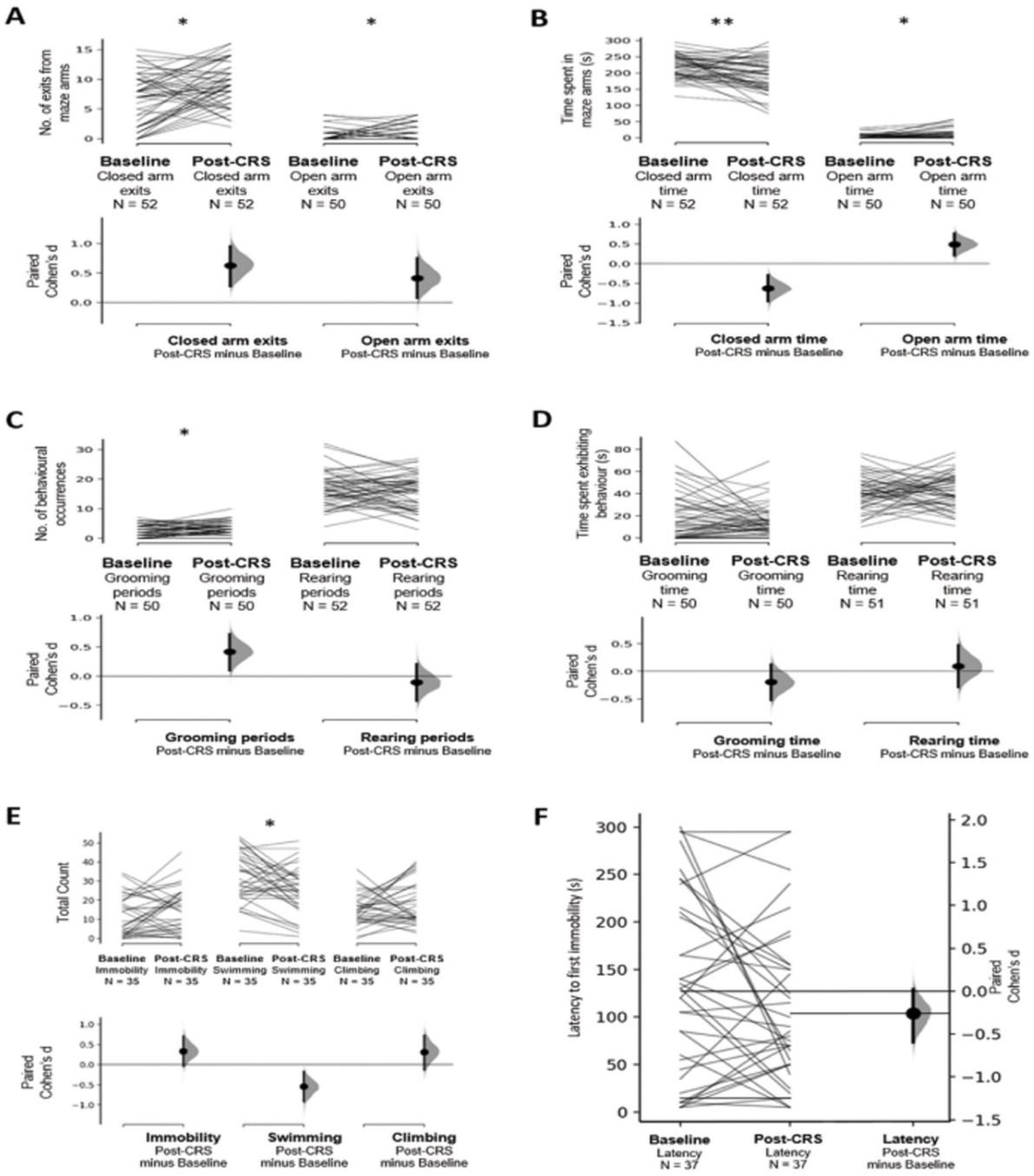

Fig. 2.

Comparisons of depression-related behaviours at baseline and post-CRS timepoints from the Elevated Plus Maze (**2a-d**) and Forced Swim Test (**2e-f**). **2a.** Total number of exits into closed and open arms (i.e. exploration behaviour). **2b.** Total time spent (sec) in closed and open arms (i.e. exploration behaviour). **2c-d.** Total number of times (**2c**) and total time spent (**2d**) exhibiting anxiety-related behaviours (grooming and rearing behaviours). **2e**. Total count of immobility, swimming, and climbing behaviours. **2f.** Time taken to exhibit the first immobility count. Raw data are plotted on the top segment of the estimation plots, with

paired data points connected via a line. Paired Cohen's d for the comparisons are shown in the bottom segment† of the Cumming estimation plots (†NB: right side in **2f**). Bootstrap sampled distributions are shown via bolded vertical lines, with the centre circle indicating the mean difference, and non-bolded ends representing error bars for the 95% CIs. * denotes significance of $p < .05$, ** denotes significance of $p < .001$.

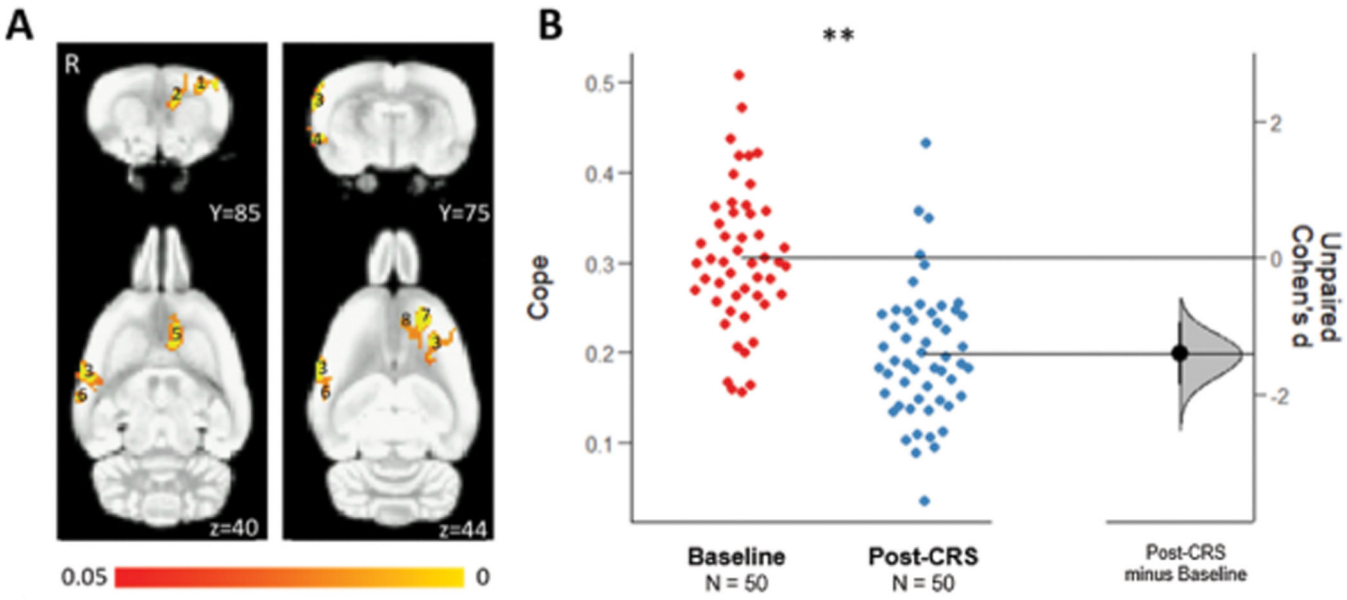

Fig. 3.

Comparisons of functional connectivity to the cingulate cortex between baseline and post-CRS timepoints assessed via seed-based analysis (**3a**) and Cumming estimation plot (**3b**). **3a**. Seed-based analysis using cingulate cortex seed. The figure displays coronal and axial slices of spatial statistical colour-coded maps overlaid on the rodent brain atlas, with resting-state networks represented as z scores ($1.96 < z < 2.58$) corrected for multiple comparisons at cluster level (thresholded at $p < .05$). The numbers on the right refer to the slice position on the atlas. R denotes right hemisphere. Significant differences were found in: 1, motor cortex; 2, cingulate cortex; 3, somatosensory cortex; 4, insular cortex; 5, infralimbic cortex; 6, auditory cortex; 7, frontal association cortex; and 8, prelimbic cortex. **3b.** Average COPE extracted from individual animals' functional connectivity to the cingulate cortex is plotted on the left of the estimation plot, with unpaired Cohen's d for the two comparisons on the right. The bootstrap sampled distribution is shown via bolded vertical lines, with the centre circle indicating the mean difference, and non-bolded ends representing error bars for the 95% CIs. ** denotes significance of $p < .001$.

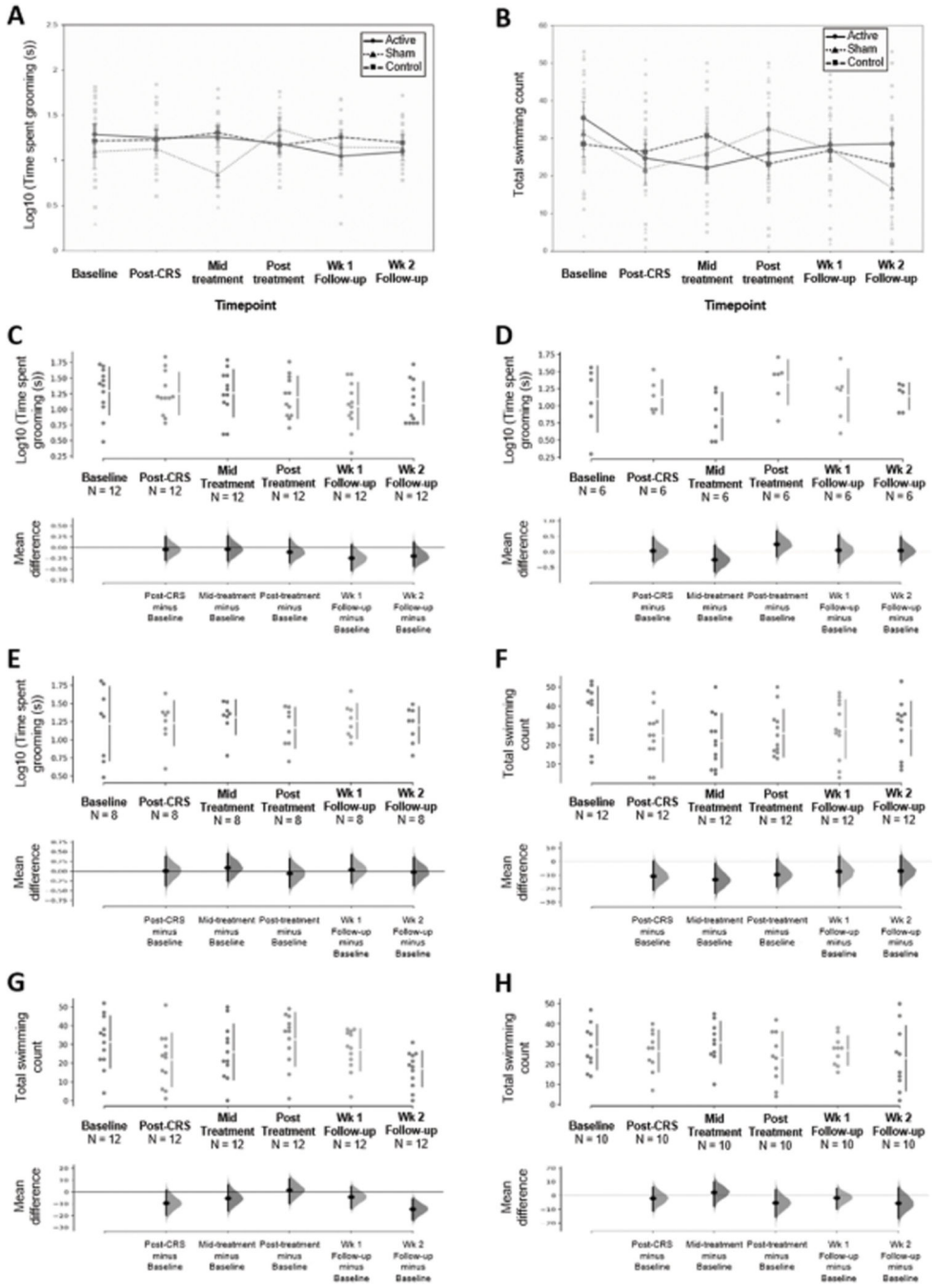

Fig. 4.

Behaviours with significant interactions from the Elevated Plus Maze (**4a, 4c-e**) and Forced Swim Test (**4b, 4f-h**) displayed across all timepoints as a line graph (**4a-b**) and Cumming estimation plots (**4c-h**). Comparison of all conditions across time for total time spent exhibiting grooming behaviour (**4a**) and total count of swimming (**4b**). Total time spent exhibiting grooming behaviour for active (**4c**), sham (**4d**) and depression control (**4e**) conditions. Total count of swimming for active (**4f**), sham (**4g**) and depression control (**4h**) conditions. Raw data are plotted on the top section of the estimation plot, with

unpaired mean difference for comparisons to baseline on the bottom. The bootstrap sampled distribution is shown via bolded vertical lines, with the centre circle indicating the average mean difference, and non-bolded ends representing error bars for the 95% CIs.

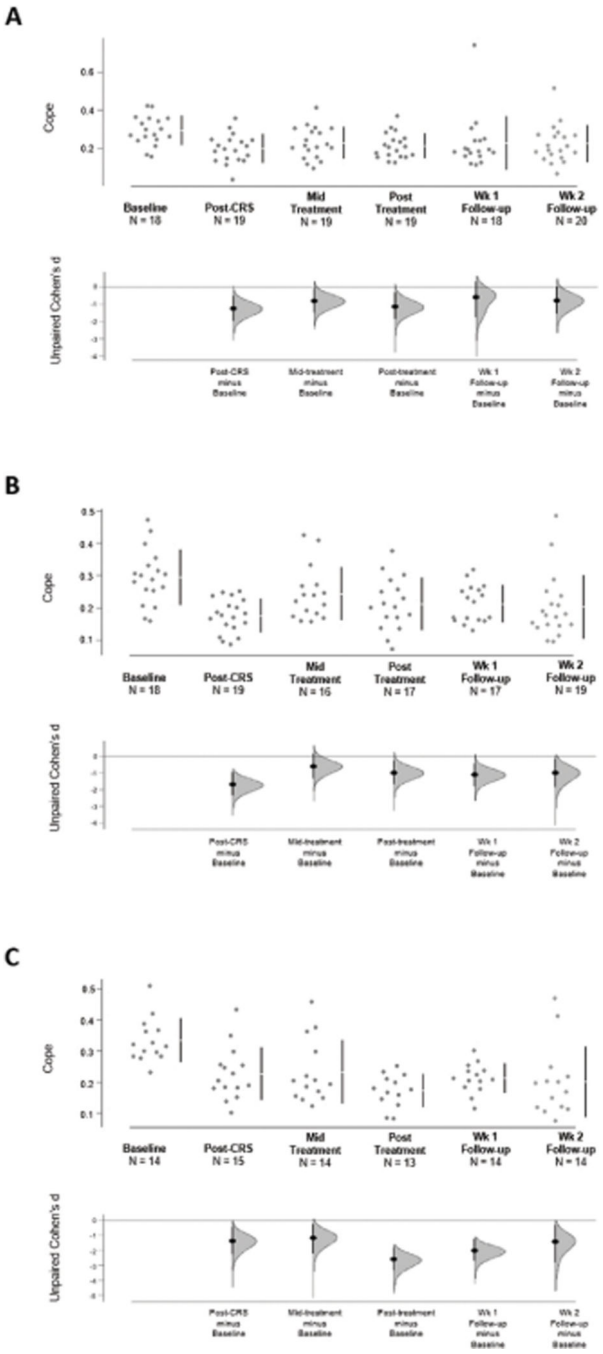

Fig. 5.

Comparisons of functional connectivity to the cingulate cortex across all timepoints for active treatment (**5a**), sham treatment (**5b**) and depression control (**5c**) conditions. Data is displayed as a Cumming estimation plot. Raw data is plotted on the top section of the estimation plot, with unpaired Cohen's d for the comparisons to baseline on the bottom. The bootstrap sampled distribution is shown via bolded vertical lines, with the centre circle indicating the mean difference, and non-bolded ends representing error bars for the 95% CIs.

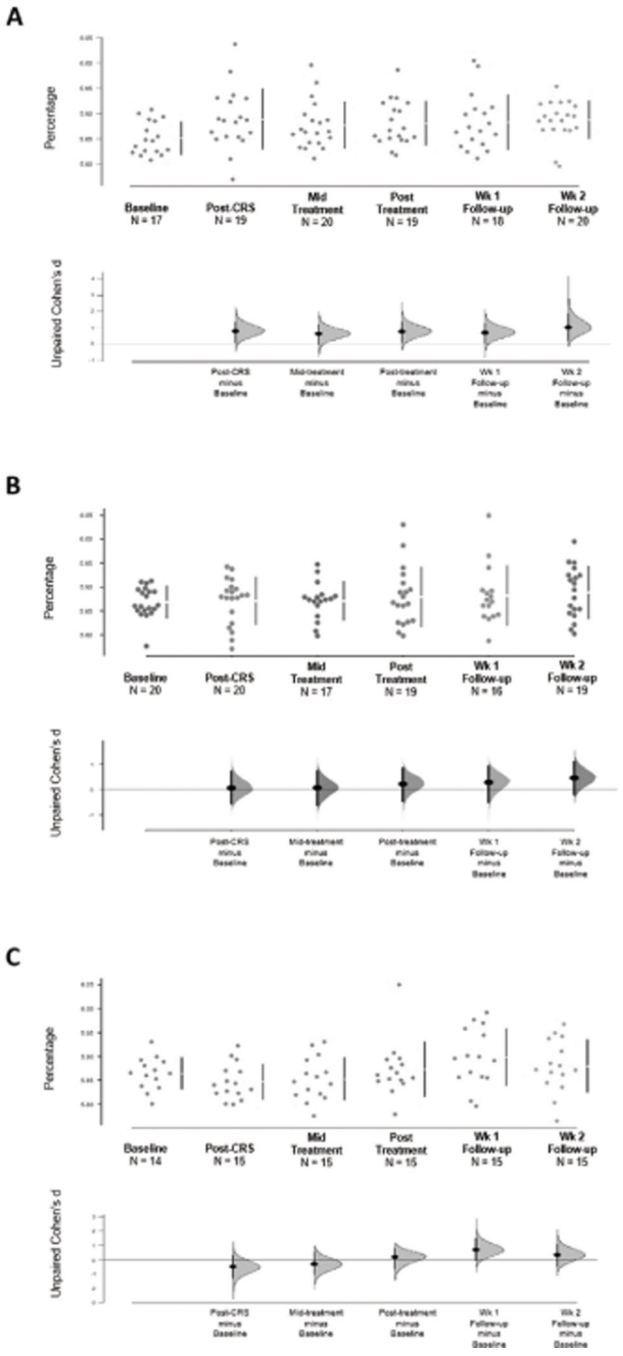

Fig. 6.

Comparisons of total hippocampal volume across all timepoints for active treatment (**6a**), sham treatment (**6b**) and depression control (**6c**) conditions. Data is displayed as a Cumming estimation plot. Raw data is plotted on the top section of the estimation plot, with unpaired Cohen's d for the comparisons to baseline on the bottom. The bootstrap sampled distribution is shown via bolded vertical lines, with the centre circle indicating the mean difference, and non-bolded ends representing error bars for the 95% CIs.

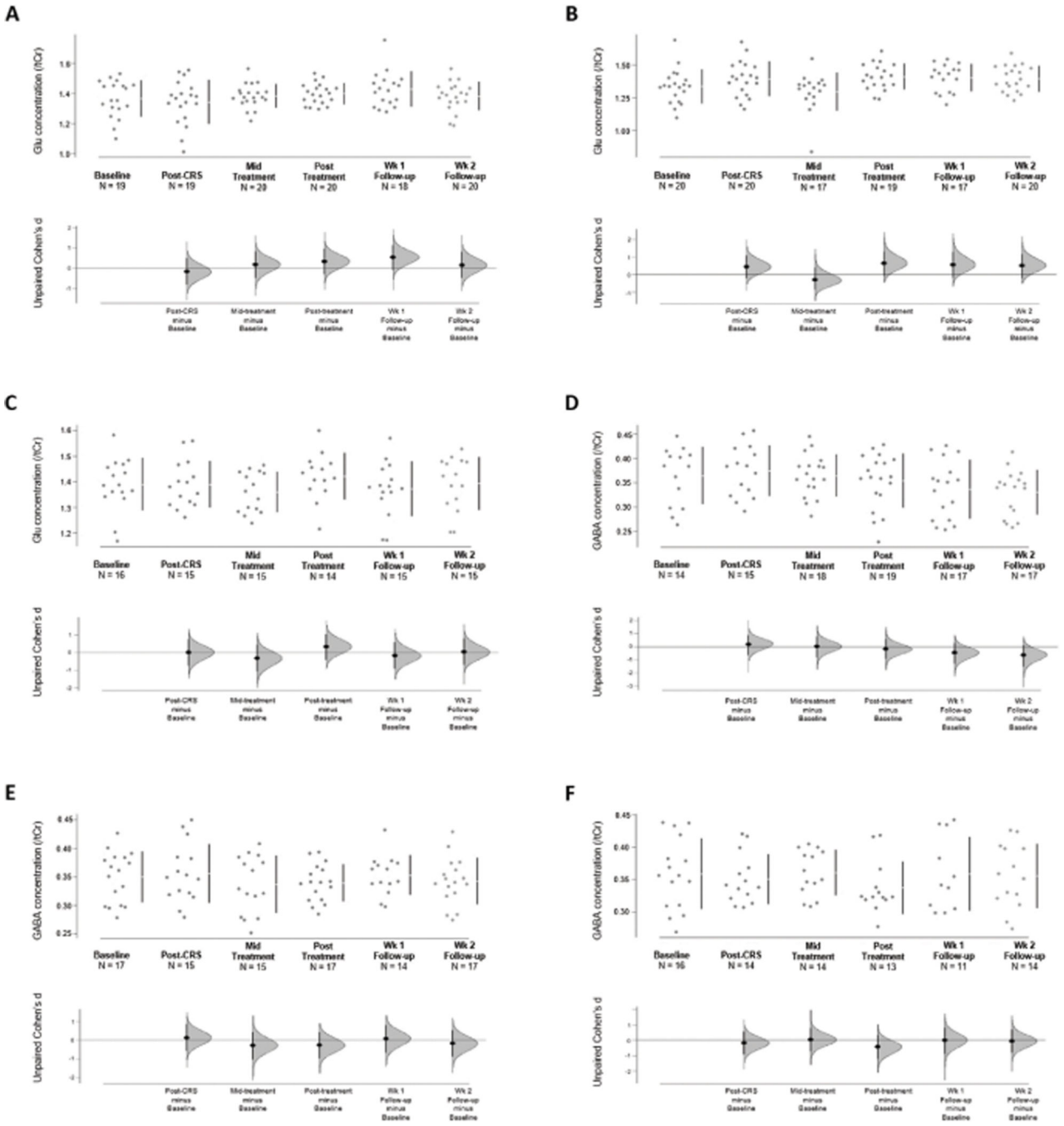

Fig. 7.

Concentration levels across all timepoints for Glutamate (Glu) (**7a-c**) and GABA (**7d-f**). Levels of Glutamate are shown within active (**7a**), sham (**7b**) and depression control (**7c**) conditions. Levels of GABA are shown within active (**7d**), sham (**7e**) and depression control (**7f**) conditions. Data is displayed as a Cumming estimation plot. Raw data are plotted on the top section of the estimation plot, with unpaired Cohen's d for the comparisons to baseline on the bottom. The bootstrap sampled distribution is shown via bolded vertical lines, with

the centre circle indicating the mean difference, and non-bolded ends representing error bars for the 95% CIs.